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(71) Applicant:
NATIONAL RESEARCH COUNCIL OF CANADA
Ottawa Ontario K1A OR6 (CA)

(72) Inventors:
- **Sung, Wing L.**
  Gloucester, Ontario K1J 6A4 (CA)
- **Yaguchi, Makoto**
  Ottawa, Ontario, K2C 3N5 (CA)
- **Ishikawa, Kazuhiko**
  Tsukuba City, Ibaragi-Ken, 305 (JP)

(74) Representative:
von Kreisler, Alek, Dipl.-Chem. et al
Patentanwälte,
von Kreisler-Selting-Werner,
Bahnhofsvorplatz 1 (Deichmannhaus)
50667 Köln (DE)

(54) Modification of xylanase to improve thermophilicity, alkophilicity and thermostability

(57) Producing a xylanase enzyme of superior performance in the bleaching of pulp. More specifically, a modified xylanase of Family 11 that shows improved thermophilicity, alkalophilicity, and thermostability as compared to the natural xylanase. The modified xylanases contain any of three types of modifications: (1) changing amino acids 10, 27, and 29 of *Trichoderma reesei* xylanase II or the corresponding amino acids of another Family 11 xylanase, where these amino acids are changed to histidine, methionine, and leucine, respectively; (2) substitution of amino acids in the N-terminal region with amino acids from another xylanase enzyme. In a preferred embodiment, substitution of the natural *Bacillus circulans* or *Trichoderma reesei* xylanase with a short sequence of amino acids from *Thermomonospora fusca* xylanase yielded chimeric xylanases with higher thermophilicity and alkalophilicity; (3) an extension upstream of the N-terminus of up to 10 amino acids. In a preferred embodiment, extension of the N-terminus of the xylanase with the tripeptide glycine-arginine-arginine improved its performance.

EP 0 828 002 A2

Descripti n

BACKGROUND OF THE INVENTION

1. *Field of the invention* The field of the invention is the modification of proteins by protein engineering. In particular, the invention concerns modified xylanase enzymes with improved performance at conditions of high temperature and pH. Xylanase enzymes are used to enhance the bleaching of pulp to make white paper. The invention enables xylanase enzymes to be produced with the benefits of enhanced bleaching associated with Family 11 xylanases, but with activity at higher temperature and pH conditions more suitable to the needs of a pulp mill's operation than xylanases currently available.

2. *Brief description of the prior art* Xylanase enzymes have been used commercially since 1991 to enhance the bleaching of pulp to make bright white paper. These enzymes are added to the pulp before the pulp is bleached, and remove a portion of the xylan in the pulp. This action allows the subsequent bleaching chemicals, including chlorine, chlorine dioxide, hydrogen peroxide, oxygen, ozone, and sodium hydroxide, to bleach the pulp more efficiently than in the absence of xylanase treatment. The enhanced efficiency of bleaching has allowed mills to reduce the amount of chlorine-based chemicals to use, which decreases the amount of toxic organochlorine compounds in the mill's effluent, as well as produce whiter pulp or allow the mill to save money on its bleaching chemicals. The commercial use of xylanase enzymes for bleaching has been reviewed by Tolan, et al, Pulp and Paper Canada, December 1995.

Xylanase enzymes have been reported from nearly 100 different microbes. The xylanase enzymes are classified into several of the more than 40 families of glycosyl hydrolase enzymes. The glycosyl hydrolase enzymes, which include xylanases, mannanases, amylases, beta-glucanases, cellulases, and other carbohydrases, are classified based on such properties as the sequence of amino acids, the three dimensional structure and the geometry of the catalytic site (Gilkes, et al, (1991) Microbiol. Reviews 55: 303-315).

Of particular interest for pulp bleaching applications are the enzymes classified in Family 11. All of these are xylanases and are known as the "Family 11 xylanases". Some publications refer to these synonymously as the Family G xylanases, but we shall use the term Family 11.

TABLE 1 lists the Family 11 xylanases known at the present time. Most of them are of molecular mass of about 21,000 Da. Three of the Family 11 xylanases-*Clostridium stercorarium* XynA, *Streptomyces lividans* XynB, and *Thermomonospora fusca* XynA-have a higher molecular mass of 31,000 to 50,000 Da. However, these xylanases have a catalytic core sequence of about 21,000 Da similar to the other Family 11 xylanases. The amino acid sequences of the Family 11 xylanases (or, for the larger enzymes, the catalytic core) show a high degree of similarity (Figure 1). The Family 11 xylanases, which are of bacterial, yeast, or fungal origin, share the same general molecular structure (see Figure 2, of CAMPBELL et al. U.S. Pat.No. 5,405,769).

TABLE 1

Family 11 xylanases	
Microbe	Xylanase
Aspergillus niger	Xyn A
Aspergillus kawachii	Xyn C
Aspergillus tubigensis	Xyn A
Bacillus circulans	Xyn A
Bacillus pumilus	Xyn A
Bacillus subtilis	Xyn A
Cellulomonas fimi	Xyn D
Chainia spp.	Xyn
Clostridium acetobutylicum	Xyn B
Clostridium stercorarium	Xyn A
Fibrobacter succinogenes	Xyn C
Neocallimastix patriciarum	Xyn A
Nocardiopsis dassonvillei	Xyn II
Ruminococcus flavefaciens	Xyn A
Schizophyllum commune	Xyn
Streptomyces lividans	Xyn B
Streptomyces lividans	Xyn C
Streptomyces sp. No. 36a	Xyn
Streptomyces thermoviolaceus	Xyn II
Thermomonospora fusca	Xyn A
Trichoderma harzianum	Xyn
Trichoderma reesei	Xyn I
Trichoderma reesei	Xyn II
Trichoderma viride	Xyn

An enzyme is classified in Family 11 if it possesses the amino acids common to Family 11, including two glutamic acid (E) residues serving as the essential catalytic residues. These E residues are amino acids 86 and 177 by *Trichoderma reesei* xynII numbering. The corresponding location of the key E residues for other Family 11 xylanases is easily determined by aligning the amino acid sequences, a procedure familiar to those skilled in the art. The amino acids common to Family 11 xylanases are indicated in bold type in Figure 1 (Wakarchuk, et al, Protein Science 3:467-475 (1994).

The Family 11 xylanases have several advantages over other xylanases in pulp bleaching applications. Most of the Family 11 xylanases are smaller than xylanases in other families. The small size relative to other xylanases is probably beneficial in penetrating the pulp fibers to release xylan from the pulp and enhance the bleaching. The Family 11 xylanases are also "pure" xylanases in terms of their catalytic activity. Unlike the xylanase enzymes in other families, these enzymes hydrolyze only xylan and do not hydrolyze cellulose. Cellulose hydrolysis damages the pulp and is unacceptable in a commercial mill. Among the Family 11 xylanases, the xylanases made by the wood-rotting fungus *Trichoderma* have been the most widely used in enhancing pulp bleaching. In particular, *Trichoderma reesei* xylanase II (Xyn II), with molecular weight 21,000 and isoelectric point 9.1, has been widely used.

In spite of the advantages of Family 11 xylanases in pulp bleaching, these enzymes have significant drawbacks. The range of temperature and pH that the enzymes exhibits activity on pulp are 45 °C to 55 °C and pH 5.0 to 7.5. A small proportion of mills have operated historically within these ranges. Typically, however, the pulp is at a temperature

of 60 °C to 70 °C and a pH of 10 to 12. In some mills the adjustment of temperature and pH are acceptable and routine. However, in many mills achieving the desired treatment conditions causes severe problems.

Depending upon how the bleaching is carried out, cooling of the pulp to temperatures below 60 °C can decrease the efficiency of bleaching to an unacceptable extent. For example, if a mill is bleaching entirely with chlorine dioxide and has a retention time of less than 20 minutes in the chlorination tower, the minimum temperature for adequate bleaching is 60 °C. If such a mill cannot heat the pulp between the enzyme treatm nt and the chlorination, which is often the case, then lower temperatures for the enzyme treatment stage are unacceptable.

Sulfuric acid is used to control the pH of the pulp. Depending on the metallurgy of the equipment, the use of sulfuric acid to control the pH can corrode the steel pipes and other equipment. Sulfuric acid is also a safety hazard.

Another minor problem with using these enzymes, and in particular *Trichoderma reesei* xylanase for bleaching applications is the low thermostability. There is the possibility that the warm ambient temperatures in the mills can inactivate the enzymes after several weeks storage. This problem is not as important as the difficulties of adjusting the temperature and pH of the pulp, but must be taken into account by using refrigerated storage or adding stabilizer compounds to the enzyme.

Therefore, the use of xylanase enzymes, particularly Family 11 xylanase enzymes, active at higher pH and temperature ranges than *Trichoderma reesei* Xyn II would be desirable. It would allow mills that operate outside of the active ranges of *Trichoderma* xylanase to be able to carry out xylanase treatment and obtain the benefits associated with the treatment. It would also allow mills to carry out xylanase treatment using less sulfuric acid and cooling water than is currently the case, saving production costs and increasing controllability and storage stability.

Before discussing the approaches that have been taken to improving the properties of xylanase enzymes, it is useful to define the following terms.

Thermophilicity is defined herein as the ability of an enzyme to be active at a high temperature. For example, xylanase #1 has more thermophilicity than xylanase #2 if it is capable of hydrolyzing xylan at a higher temperature than xylanase #2. Thermophilicity relates to enzyme activity in the presence of substrate. In the present invention, the substrate can be pulp xylan or purified xylan.

It is important to specify the substrate for purposes of defining the thermophilicity. Most xylanase enzymes are effective at higher temperatures in the hydrolysis of pure xylan than in the treatment of pulp. This is due to a combination of factors relating to the substrates (i.e. inhibitors present in the pulp) and to the length of time, pH, and other aspects of the procedures used to carry out the tests. Quantitative measures of thermophilicity refer herein to pure xylan substrates unless otherwise indicated.

Thermostability is defined herein as the ability of an enzyme to be stored incubated at a high temperature in the absence of xylan substrate, and then exhibit xylanase activity when returned to standard assay conditions. For example, xylanase #1 is more thermostable than xylanase #2 if it can be held at 70°C for 24 hours and retain all of its activity, while xylanase #2 loses all of its activity after 24 hours at °70 C. In contrast to thermophilicity, thermostability relates to the enzyme activity remaining after incubation in the absence of xylan substrate.

These two terms are defined explicitly to overcome confusion in the literature, where the two terms are often used synonymously or to denote each other. Their present usage is consistent with Mathrani and Ahring, Appl. Microbiol. Biotechnol. 38:23-27 (1992).

Alkalophilicity is defined herein as the ability of an enzyme to be active at a high (alkaline) pH. For example, xylanase #1 has more alkalophilicity than xylanase #2 if it is capable of hydrolyzing xylan at a higher pH than xylanase #2. Alkalophilicity is analogous to thermophilicity and relates to enzyme activity in the presence of xylan substrate.

For improving xylanase for pulp bleaching applications, the thermophilicity and alkalophilicity are much more important than the thermostability. Most of the work described in the prior art has focussed only on improving the thermostability.

Two generic approaches can be taken to make xylanase enzymes with higher pH and temperature ranges. These are: (1) screening naturally-occurring xylanase enzymes with the desired properties, and (2) using protein engineering to improve the properties of existing xylanase enzymes.

Among naturally occurring xylanases, thermostable enzymes have been isolated from thermophilic microbes, such as *Caldocellum saccharolyticum*, *Thermatoga maritima* and *Thermatoga* sp. strain FjSS3-B.1, all of which grow at 80-100°C (Lüthi et al. 1990; Winterhalter et al. 1995; Simpson et al. 1991). However, all are relatively large in size with high molecular mass of 35-120 kDa (320-1100 residues). Some of these xylanases (*C. saccharolyticum* xylanase A) belong to families other than Family 11 and have both xylanase and cellulase activities (Lüthi et al. 1990). Such cellulase activity is undesirable for pulp bleaching. Furthermore, hyperthermostable xylanases which function normally at extremely high temperatures have low activity at the comparatively lower temperatures for pulp bleaching.

Most of the Family 11 xylanases are effective in pulp bleaching applications at 45 °C to 55 °C. However, Family 11 also includes at least two thermostable xylanases, both of which happen to have a higher molecular mass than the other Family 11 xylanases. These xylanases are *Thermomonospora fusca* xylanase (known as TfxA) of 296 amino acids and 32,000 Da (Irwin et al.(1994) Appl. Environ. Microbiol. 60:763-770; Wilson et al. 1994, WO 95/12668) and

Clostridium stercorarium xylanase A which is of 511 amino acids and 56,000 Da with an optimum temperature of 70°C (Sakka et al. (1993) Biosci. Biotech. Biochem. 57:273-277).

These thermostable xylanase enzymes have some features that are potential problems in pulp bleaching applications. First, the large molecular weight might limit the penetration of the enzymes into the pulp fibers. Second, these enzymes have at least a single copy of a cellulose binding domain (CBD) not present in the other Family 11 xylanases. The CBD, located in the extended C-terminus of TfxA, causes precipitation of the protein and loss of activity in storage.

Therefore, those naturally occurring xylanase enzymes have limitations.

An alternative approach is to carry out protein engineering of a well-known xylanase enzyme. By using protein engineering, specific changes can be made to the protein which might improve a desired property, such as temperature or pH range, without compromising on secondary properties such as protein solubility.

When carrying out protein engineering to modify protein properties, one must select the general method to use and then the specific sites and modifications to make. The general methods include (1) site-specific mutagenesis, (2) random mutagenesis, (3) chimeric modification, (4) dimerization, and (5) glycosylation. Within each of these general methods, there are an enormous number of options of specific modifications to the protein that one can make. The effects of different mutations on enzyme characteristics, including thermophilicity and alkalophilicity, are often unpredictable. Generally, only a tiny fraction of all possible modifications, if any, provide significant benefit. Therefore, setting out to improve the properties of a protein by protein engineering is a difficult venture, and the limited success to date with Family 11 xylanases reflects this. The work with modified xylanases is described as follows.

Site-specific mutagenesis involves the modification of specific amino acids in a protein. The modifications based on site-specific mutagenesis are known as point mutations, Site-specific mutagenesis of Family 11 xylanases has been used to produce xylanase enzymes of slightly improved thermostability. CAMPBELL et al, (U.S. Pat. No. 5,405,769) described one manner of improvement of *Bacillus circulans* xylanase (abbreviated BcX), a xylanase of Family 11, through two types of modifications. These were (i) intramolecular disulfide bonds, and (ii) site-specific mutations at the N-terminus.

CAMPBELL et al, describes how disulfide bonds may be inserted between amino acids #98 and #152, #100 and #148, and # (-1) and #187, according to the amino acid numbering of *Bacillus circulans* xylanase. The disulfide modifications improved the thermostability of the xylanase at 62°C. However, these disulfide-modified enzymes showed no gain in thermophilicity (Wakarchuck et al. (1994) Protein Engineering 7:1379-1386). Therefore, thermostability and thermophilicity are not necessarily coupled.

CAMPBELL et al, also describes three modifications (designated T3G, D4Y(F) and N8Y(F)) near the N-terminus of BcX generated mutant xylanase with thermostability at 57°C, a small increase of 2° C. In the PCT publication WO 94/24270, which is related to CAMPBELL et al, there is a description of a fourth advantageous modification, S22P, for the improvement of BcX. This set of four modifications (designated TS19a in the document) showed a higher thermostability and thermophilicity than BcX. However, certain factors would limit the application of these modifications in Family 11 xylanases other than BcX. These mutations to convert residues-3, 4, 8 and 22 (BcX amino acid numbering) respectively into Glycine, Tyrosine (or Phenylalanine), Tyrosine (or Phenylalanine) and Proline, respectively, are irrelevant to the majority of the Family 11 xylanases, as they already possess these "good" residues (see Figure 1). The best illustration of the inadequacy of these modifications is Xyn II of *Trichoderma reesei*, which possesses all four "good" residues, yet is mediocre in thermophilicity and alkalophilicity.

Random mutagenesis involves the modification of amino acids at random within the entire protein. This method was used to produce a Family 11 xylanase with improved thermostability by ARASE et al ((1993) FEBS Lett. 316:123-127), which described modest improvement of thermostability of a *Bacillus pumilus* xylanase (abbreviated BpX) through modifications at residues-12, 26, 38, 48 and 126 (according to the BpX amino acid numbering). However, ARASE et al did not report any improvement in the thermophilicity or alkalophilicity as a result of their particular modifications. The gain in thermostability by the most improved ARASE et al example in a BpX xylanase was small, only allowing the maintenance of 40% of the residual enzymatic activity after incubation at 57°C for 20 min. For two other BpX xylanases, with the modifications of residues 12 and 26 around the N-terminus, the gain in thermostability represented the maintenance of 1 and 11% residual activity after incubation, respectively. Furthermore, the BpX xylanase with the residue 26 modification has other modifications as well, so the contribution of this sole modification to thermostability, if any, is unclear from ARASE et al .

A chimeric modification involves substituting some of the amino acids of a protein with a sequence of amino acids from another protein. To our knowledge, such an approach has not been carried out with any Family 11 xylanases.

Dimerization involves combining two molecules into a single protein. This technique has been used to link two BcX molecules via an intermolecular disulfide bond (Wakarchuk, et al, Protein Engineering (1994)). The resulting dimeric BcX showed only an insignificant gain in thermostability, much less than BcX with an intramolecular disulfide bond described above.

It is well known that natural glycosylation, the attachment of carbohydrates to a protein, sometimes improves the thermostability of proteins, including in *Trichoderma reesei* xyn II. Synthetic glycosylation has not been used to improve

these properties in a Family 11 xylanase.

No matter which method of protein engineering is used, a key aspect is determining which amino acids to modify, because few choices will improve the properties of the enzyme. This point is illustrated by the work of Sung, et al, Biochem. Cell Biol. 73:253-259 (1995), who modified amino acid #19 in *Trichoderma reesei* xylanase II from asparagine to lysine. This modification decreased the thermophilicity of the enzyme by 3°C.

Therefore, in spite of a large amount of effort with Family 11 xylanases, there has not yet been a modified Family 11 xylanase produced with significantly improved thermophilicity and alkalophilicity. Such an enzyme, and in particular an engineered version of *Trichoderma reesei* xyn II, would have immediate application to the commercial process of producing bleached pulp with decreased requirements for bleaching chemicals while meeting the process conditions of the mills. Such an enzyme would also have potential application in other areas. Some examples of these are as animal feed additives to aid in the digestibility of feedstuffs, where high temperature pelleting makes current enzymes unsuited in many cases; and the processing of wheat and corn for starch production, in which the high temperatures destroy current enzymes.

SUMMARY OF THE INVENTION

The present invention relates to modifying certain specific Family 11 xylanases so as to improve thermophilicity, alkalophilicity, and thermostability. The invention has particular utility in creating enzymes that will allow a pulp mill to obtain the benefits of enhanced bleaching, known to be associated with Family 11 xylanases, but at much higher temperatures and at pH conditions which are more suitable to preferred mill operation parameters, than any xylanases currently available.

The utility of the present invention is specific to Family 11 xylanase enzymes having the following, two essential characteristics:

(i): The enzyme is made by *Trichoderma*, *Bacillus*, *Aspergillus* or *Streptomyces*
(ii): The enzyme comprises the amino acid tyrosine or phenylalanine in position 14 by *Trichoderma reesei* xylanase II numbering, or an equivalent position according to the conventional numbering used to designate other xylanases in enzymes of category (i).

For Family 11 xylanases possessing both of these characteristics, either of the following two types of modifications are taught to suprisingly increase thermophilicity, alkalophilicity, and thermostability of the enzyme:

(1) SITE-SPECIFIC MUTAGENESIS : For those selected xylanases with at least 8 amino acid residues in the N-terminus upstream from position 10 (as per *Trichoderma reesei* xylanase II numbering), the modication comprising a substitution of amino acid 10 with another amino acid. A preferred embodiment is to also substitute amino acids 27 and 29 with valine, methionine, isoleucine or leucine, in addition to the essential step of substituting amino acid 10 with a different amino acid. A most preferred embodiment is to substitute histidine, methionine, and leucine, respectively, for the naturally occuring amino acids which are found at positions 10, 27 and 29.
(2) CHIMERIC MODIFICATION : Replacing a sequence of amino acids in the N-terminal region with an equivalently positioned sequence from *Thermomonospora fusca* xylanase A (Tfx) to form a chimeric enzyme. A preferred embodiment extends the chimeric enzyme upstream from the N-terminus with a tripeptide of Glycine-Arginine-Arginine or a sequence of up to 10 amino acids from the N-terminus of *Clostridium acetobutylicum* (CaX).

Surprisingly, the xylanases modified according to the invention have much improved thermophilicity, alkalophilicity, and thermostability over counterpart unmodified enzymes. Some of these modified xylanases have been found to exhibit up to a 28 °C improvement in thermophilicity, and a 2 pH unit improvement of alkalophilicity over the natural xylanase. Furthermore, the ability of some of the modified xylanases to function at 85°C and pH 9 is significantly better than any of the confirmed thermophilic Family 11 xylanases, including TfxA.

The inventors believe that no modification of a Family 11 xylanase has previously been reported with such suprising improvements in the thermophilicity, alkalophilicity, and thermostability of the Family 11 xylanases .

The modified *Bacillus circulans* xylanases of the present invention in particular are active at much higher temperatures than the 60-70°C temperature ranges disclosed for those modified *Bacillus* xylanases illustrated within ARASE et al. (BpX therein) and CAMPBELL et al. (BcX therein).

Furthermore, the modified xylanases of the invention demonstrate suprisingly improved properties for the problem of treatment of pulp. The modified xylanases also exhibit the overall potency in treating pulp that is typical of Family 11 xylanases at optimum conditions, but not observed with other xylanases.

Protein modifications as taught herein have not been previously reported for Family 11 xylanases, and no prior disclosures have suggested that the improvements as taught herein might be able to increase thermophilicity, alkalophilic-

ity, or thermostability.

ARASE, et al ((1993) FEBS Lett. 316:123-127) described modest improvement of thermostability of a *Bacillus pumilus* xylanase (abbreviated BpX) through modifications at residues-12, 26, 38, 48 and 126, according to the BpX amino acid numbering. These correspond to *Trichoderma reesei* xynII residues 11, 26, 38, 48, and 121, respectively, and not residues according to the principles of the present invention. Furthermore, unlike the present inventors, Arase did not report any improvement in the thermophilicity or alkalophilicity as a result of their teachings. The gain in thermostability by the most improved Arase BpX was small, only allowing the maintenance of 40% of the residual enzymatic activity after incubation at 57°C for 20 min. For two other BpX xylanases, with the modifications of residues 12 (11 in xynII) and 26, the gain in thermostability represented the maintenance of 1 and 11% residual activity after storage, respectively. Furthermore, the BpX xylanase with the residue 26 modification has other modifications as well, so the contribution of this sole modification to thermostability, if any, was unclear.

CAMPBELL, et al., as well as the CAMPBELL, et al PCT publication, illustrate four modifications (designated T3G, D4Y(F), N8Y(F), and S22P) that might be made to *Bacillus circulans* xylanase (BcX). These changes correspond to amino acids 12, 13, 17, and 31 by *Trichoderma reesei* xylanase numbering. The amino acids taught by CAMPBELL, et al already exist in *Trichoderma* xylanase and most of the other Family 11 xylanases, and are therefore are deemed essentially irrelevant toward improving the performance of these xylanases.

The CAMPBELL, et al modifications to *Bacillus circulans* xylanase also do not improve enzyme performance nearly as much as the modified *B. circulans* xylanases of the present invention. Examples 6 and 10 show that the modified *Bacillus circulans* xylanases of the present invention have much higher thermophilicity (+14 °C) and alkalophilicity (+1.5 pH unit) than the best of the CAMPBELL, et al xylanases. In addition, at optimum temperature, the modified *Bacillus* xylanases of the present invention have three fold higher activity than that of CAMPBELL, et al.

Amino acids 10, 27, and 29 have not been previously suggested to be important to the performance of the Family 11 xylanase enzymes. Suprisingly, the inventors have shown that modification of these amino acids in *Trichoderma* xyn II to histidine, methionine, and leucine, respectively, significantly increases the thermophilicity, alkalophilicity, and thermostability of the enzyme.

It should be noted that while three of the naturally-occurring Family 11 xylanases have these particular amino acids in these positions. (xylanases produced by *Bacillus pumulis*, *Clostridium stercorarium* (xyn A), and *Thermomonospora fusca*.), none of those three xylanases exhibit the combination of desirable characteristics taught to result from following the present invention. There is no reason to expect that the thermophilicity is due to the presence of these three amino acids, and the natural xylanases do not point to these three amino acids as key to the enzyme performance. While the *Clostridium* and *Thermonospora* xylanases are thermophilic, the *B. pumilis* xylanase is not thermophilic, as it has an optimum temperature below 40°C (Nissen, et al, 1992). In addition to the three common positions mentioned, there are over 75 positions with identical amino acids in the three xylanases. Both of the *Clostridium* and *Thermonospora* xylanases contain unique cellulose binding domains which have been postulated to confer thermostability, as in the other Families of xylanases (Fontes, et al, 1995, Biochem. J. 307:151-158).

The inventors do not purport to claim any of the three natural Family 11 xylanases that possess the preferred residues in positions 10, 27, and 29. The invention is restricted to those xylanases with tyrosine or phenylalanine in position 14 by *T. reesei* xyn II, or the corresponding position in other xylanases. This excludes the *B pumilis* xylanase, which has aspartic acid in this position 14. The invention is also restricted to xylanases made by *Trichoderma*, *Streptomyces*, *Aspergillus*,, and *Bacillus*. This excludes the *Thermomonospora* and *Clostridium* xylanases.

In addition to these three xylanases, two others in Family 11 have methionine in position 27 and leucine in position 29. These are *Clostridium acetobutylicum* xynB (optimum temperature 43 °C) and *Streptomyces lividans* xynC (optimum temperature 55 °C). Neither of these enzymes, however, are thermophilic and therefore neither suggests the modifications to positions 27 and 29 will be useful.

The specific choices of histidine, methionine, and leucine in positions 10, 27, and 29 improves the stability of *Trichoderma reesei* xynII Given, this fact, it will now be recognized by those skilled in the art that any of a number of amino acids substituted into position 10 will improve the properties of the enzyme. It will also be recognized that, given the improvements due to methionine and leucine in positions 27 and 29, any of the hydrophobic, medium sized amino acids, including valine, isoleucine, leucine, and methionine will be beneficial in these positions.

The important discovery in this aspect of the invention is that positions 10, 27, and 29 are important for the stability of *Trichoderma reesei* xynII. Based on this teaching, it will be appreciated by those skilled in the art that this modification will be beneficial to certain other Family 11 xylanases, and that Family 11 xylanases must satisfy two conditions to benefit from this modification.

First, the Family 11 xylanase must possess at least eight amino acid residues upstream from position 10. Several Family 11 xylanases have a truncated N-terminus. The modification to position 10 is not relevant to xylanases with a truncated N-terminus.

Second, the Family 11 xylanase must possess the amino acid tyrosine or phenylalanine in position 14 by *Trichoderma reesei* xylanase II numbering, or the corresponding position in other xylanases. The side chain of tyrosine or phe-

nylalanine at position 14 points directly into the active site of the protein. Tyrosine and phenylalanine are of similar size and each has a six member aromatic ring that potentially may participate in a stacking interaction with the xylose ring when bound to a xylan substrate. The presence of other amino acids in this position causes significant changes to the overall structure of the protein. Therefore, only those Family 11 xylanase enzymes with either of these amino acids in position 14 will be suitable for modifications to position 10, while Family 11 xylanases with other residues in the corresponding position will not be amenable to this modification.

Of the known Family 11 xylanases of *Trichoderma*, *Streptomyces*, *Bacillus*, and *Aspergillus*, only *Trichoderma reesei* xyn II, *Trichoderma harzianum* xyn, *Trichoderma viride* xyn, *Streptomyces lividans* xyn B, and *Streptomyces lividans* xyn C meet these two conditions. Therefore, these are the only enzymes presently known that are suitable for the modifications to positions 10, 27, and 29 taught herein.

The chimeric xylanases of the invention have not been previously reported, and there is nothing published to suggest that these particular chimeric xylanases would be beneficial in xylanase performance. Even more surprisingly, in some cases, the thermophilicity and alkalophilicity of the chimeric xylanases is better than that of either of the individual xylanases that comprise the chimeric xylanase. Examples of these enzymes are the modified xylanases NI-BX6 and NI-BX7 in Examples 7 and 11.

The important discovery in this aspect of the invention was that chimeric modification of segments of the N-terminal region of *Trichoderma reesei* xylanase II or *Bacillus circulans* xylanase A with *Thermonospora fusca* xylanase A could increase the thermophilicity, alkalophilicity, and thermostability of the enzymes.

Based on this teaching, it will be recognized by those skilled in the art that chimeric xylanases with improved performance can be formed consisting of the Family 11 xylanases from other *Trichoderma*, *Aspergillus*, *Streptomyces*, and *Bacillus*, strains with *Thermomonospora fusca* xyn A provided that one condition is met. The Family 11 xylanase must possess the amino acid tyrosine or phenylalanine in position 14 by *Trichoderma reesei* xylanase II numbering, or the corresponding position in other xylanases. The side chain of tyrosine or phenylalanine in position 14 points directly into the active side of the protein. Tyrosine and phenylalanine are of similar size and their six member aromatic ring can potentially participate in a stacking interaction with the xylose ring when bound to the xylan substrate. *T. fusca* xylanase A has phenylalanine in this position. Only those Family 11 xylanase enzymes with either tyrosine or phenylalanine in position 14 will be suitable for forming chimeric xylanases with *T. fusca* xylanase A. Family 11 xylanases with other residues in the corresponding position will not be amenable to this modification.

Of the known Family 11 xylanases of *Trichoderma*, *Streptomyces*, *Bacillus*, and *Aspergillus*, only *Trichoderma reesei* xyn II, *Trichoderma harzianum* xyn, *Trichoderma viride* xyn, *Trichoderma reesei* xyn I, *Streptomyces lividans* xyn B, *Streptomyces lividans* xyn C, *Bacillus circulans* xyn A, *Bacillus subtilis* xyn A, *Aspergillus niger* xyn A, *Aspergillus kawachii* xyn A, and *Aspergillus tubigensis* xyn A meet this condition. Therefore, these are the only enzymes presently known that are suitable for the chimeric modifications taught herein.

Upstream extension of the protein has never been reported to enhance the performance of the Family 11 xylanase enzyme, nor has it been reported to be beneficial in other enzymes. There is no reason to expect that adding amino acids upstream of the N-terminus would improve the thermophilicity, alkalophilicity, or thermostability.

There is no reason to expect that upstream extension with the three amino acids glycine-arginine-arginine would improve the performance of the enzyme. This set of three amino acids is not found in any natural xylanases. Two of the natural Family 11 xylanases, *Bacillus pumulis* and *Clostridium stercorarium*, have only a single arginine directly upstream of the N-terminus. Both xylanases do not have the other arginine and glycine which are essential to the improved thermophilicity of both modified BcX and TrX. The *Clostridium* enzyme is thermophilic, but the *Bacillus* enzyme is not, so the natural enzymes do not point to this modification.

There is no reason to expect that upstream extension of the N-terminus with a 10 amino acid sequence from *Clostridium acetoburylicum* xyn B (CaX) would improve the thermophilicity of other Family 11 xylanases. CaX has an optimum temperature of only 43 °C, which is no better than the other Family 11 xylanases. Therefore, it is surprising that a sequence of amino acids from this enzyme would improve the thermophilicity of these other xylanases.

The upstream extensions described herein were demonstrated on chimeric xylanases formed by substituting an N-terminal sequence from *T. fusca* xylanase A into xylanase II from *T. reesei* and xylanase A from *B. circulans*. It will be recognized by those skilled in the art that said upstream extension will improve the performance of chimeric xylanases that be can be formed by substituting an N-terminal sequence from *T. fusca* xylanase A into a Family 11 xylanase from *Trichoderma*, *Aspergillus*, *Streptomyces*, and *Bacillus*.

In summary, the inventors have developed unique enzymes with desirable properties for commercial applications to treating pulp to improve its bleaching.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the multiple amino acid sequence alignment among several Family 11 xylanases. Each letter represents an amino acid, with the standard amino acid abbreviations used. The divisions into sections are chosen arbitrarily

to fit the typeset of the page and have no relevance to the structure of the proteins. The 1 to 30 amino acid numbering for *Trichoderma reesei* xyn Ii is indicated. The amino acids common to at least 80% of the Family 11 xylanases listed are indicated in bold. The residues common to all Family 11 xylanases are underlined. For xylanases of *Clostridium stercorarium*, *Streptomyces lividans* (xynB), and *Thermomonospora fusca*, only the catalytic core sequences are presented.

FIG. 2 shows the main chain structures of the fungal *Trichoderma harzianum* xylanase (ThX) and the bacterial *Bacillus circulans* xylanase (BcX).

FIG. 3 shows the synthetic oligonucleotides for the construction of the gene sequence encoding the *Trichoderma* xylanase in the plasmid pTvX(3-190).

FIG. 4 shows the synthetic oligonucleotides for the construction of the gene sequence encoding the *Bacillus circulans* xylanase BcX in the plasmid pXYbc.

FIG. 5 shows the effect of temperature on the enzymatic activity of NI-TX mutant xylanases. Enzymatic activity was normalized to that at 40 °C.

FIG. 6 shows the effect of temperature on the enzymatic activity of NI-BX mutant xylanases. Enzymatic activity was normalized to that at 40°C. The profile of BcX mutant TS19a of Campbell, et al is also presented.

FIG. 7 shows the effect of pH on the enzymatic activity of NI-TX modified *Trichoderma* xylanases at 65°C. The data are normalized to the maximum enzymatic activity.

FIG. 8 shows the effect of pH on the enzymatic activity of NI-BX modified xylanases at 65 °C. The profile of BcX mutant TS19a is also presented. The data are normalized to the maximum enzymatic activity.

FIG. 9 shows the effect of pH on the enzymatic activity of the modified *Bacillus* xylanases at 50 °C. Data on TfxA published by Wilson et al (PCT, 1995) is included. For the purpose of comparison with TfxA, the enzymatic activity of all modified *Bacillus* xylanases was normalized to the pH 8 results.

FIG. 10 shows the thermostability of modified *Trichoderma* xylanases NI-TX1, NI-TX5, NI-TX10, NI-TX11, TvX(3-190) and the natural TrX at 53°C. Enzymatic activity was normalized to that at 0 min of incubation.

FIG. 11 shows the thermostability of chimeric xylanases NI-TX2, NI-TX5, NI-TX8, NI-TX9 and natural TrX at 68°C. The profile of natural TrX at 53 °C was also included for comparison. Enzymatic activity was normalized to that at 0 min of incubation.

FIG. 12 shows the thermostability of NI-BX and BcX at 70°C. Enzymatic activity was normalized to that at 0 min of incubation.

FIG. 13 shows the effect of temperature on the performance of NI-TX xylanase, recombinant and natural TrX in enhancing the bleaching of pulp (tests carried out by Iogen Corporation).

FIG. 14 shows the effect of temperature on the performance of NI-BX1 and wild-type BcX in enhancing the bleaching of pulp (tests carried out by Iogen Corporation).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention comprises modified Family 11 xylanase enzymes that exhibit enhanced properties that are important in commercial pulp bleaching applications, namely thermophilicity, alkalophilicity, and thermostability. The modified xylanases exhibit enhanced properties relative to the natural enzymes. The natural enzymes are selected from the group consisting of Family 11 xylanases from *Trichoderma*, *Bacillus*, *Streptomyces*, and *Aspergillus*. The selection of natural xylanases is further restricted to those xylanases with tyrosine or phenylalanine in position 14 corresponding to the amino acid numbering in *Trichoderma reesei* xylanase II, or the equivalent position in other Family 11 xylanases.

The modifications to the selected xylanase comprise either or both of the following, which are described herein by the amino acid numbering of *Trichoderma reesei* xylanase II and apply to the corresponding aligned amino acids of the other selected Family 11 xylanases:

(1) For selected xylanases with at least 8 amino acid residues in the N-terminus upstream from position 10, substitution of amino acid 10 with another amino acid.

(2) Substitution of a sequence of amino acids in the N-terminal region with an equivalently positioned sequence from *Thermomonospora fusca* xylanase A (Tfx) to form a chimeric xylanase, and extending the protein upstream of the N-terminus with a sequence of up to 10 amino acids from another xylanase.

The invention is concerned with modified xylanases and does not claim naturally-occurring xylanases.

In practicing the invention, the starting point is a Family 11 xylanase. An enzyme is classified in Family 11 if it possesses the amino acids common to Family 11, including two glutamic acid (E) residues serving as the essential catalytic residues. These E residues are amino acids 86 and 177 by *Trichoderma reesei* xyn II numbering. The corresponding location of the key E residues for the other Family 11 xylanases is easily determined by aligning the amino acid

sequences, a procedure familiar to those skilled in the art. The amino acids common to the Family 11 xylanases are indicated in bold type in Figure 1. (Wakarchuck, et al, Protein Science 3:467-475 (1994).

The natural Family 11 xylanase used to practice the invention must be among from those Family 11 xylanases made by *Trichoderma*, *Bacillus*, *Streptomyces*, and *Aspergillus*. The enzyme must also have tyrosine or phenylalanine in position 14 by *Trichoderma xylanase II* numbering, or the corresponding position in other xylanases. The amino acid numbering for *Trichoderma reesei* xyn II is shown in Figure 1.

The substitution of amino acid 10 refers to this amino acid of *Trichoderma reesei* xylanase II. This modification is claimed only for those selected xylanase enzymes that possess at least 8 amino acid residues upstream of the position corresponding to amino acid 10 in the N-terminus.

In a preferred embodiment, the selected xylanase for this modification consists of *T. reesei* xyn II, *T. harzianum* xyn, *T. viride* xyn, *S. lividans* xyn B, or *S. lividans* xyn C. In a preferred embodiment, in addition to substitution of amino acid 10 with another amino acid, amino acids 27 and 29 are substituted with methionine, isoleucine, leucine, or valine. As with amino acid 10, amino acids 27 and 29 are identified using the *Trichoderma reesei* xylanase II numbering illustrated in Figure 1.

In a more preferred embodiment, amino acids 10, 27, and 29 are substituted for histidine, methionine, and leucine, respectively. The modified xylanases of this type are identified as NI-TX11, NI-TX12 and NI-TX13 in the Examples.

The chimeric modification of the xylanase consists of removing a sequence of amino acids from the N-terminal region of the selected xylanase and replacing with a sequence of amino acids from the N-terminal region of *Thermomonospora fusca* xylanase A.

As used herein to describe the present invention, the term "N-terminal region" refers to the first 31 amino acids of the *Trichoderma reesei* xylanase II protein closest to the N-terminus. For other Family 11 xylanases, the N-terminal region consists of the amino acids corresponding to the first 31 amino acids of *Trichoderma reesei* xylanase II when the sequences are aligned. The general definition of N-terminal region used in the art is the first 1/3 of the protein closest to the N-terminus. The definition of N-terminal region used in the present invention is consistent with, but necessarily more precise, than the general definition.

The replacement of the amino acids that have been removed is by an amino acid sequence located in the same position relative to the N-terminus of the xylanases from the two microbes. Where the two xylanases have different numbers of amino acids, the replacement sequence of amino acids is at the location that coincides with the original sequence of amino acids when the amino acid sequences of the two enzymes are aligned so as to match as closely as possible. This alignment of amino acids is familiar to those skilled in the art and is illustrated for some of the Family 11 xylanases in Figure 1.

In a preferred embodiment, the sequence of amino acids 10 to 29 from TrX or equivalent aligned amino acid sequence of another Family 11 xylanase is substituted by the corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A. An example of this modified xylanase is identified as NI-TX4 in the Examples.

In a preferred embodiment, the sequence of amino acids 1 to 29 from TrX or equivalent aligned amino acid sequence of another Family 11 xylanase is substituted by the equivalent aligned sequence of amino acids from *Thermomonospora fusca* xylanase A. An example of this modified xylanase is identified as NI-TX3 in the Examples.

In another preferred embodiment, the 1 to 22 amino acid sequence of *Bacillus circulans* xylanase (BcX) is substituted for the 1 to 31 amino acid sequence of Tfx. These two sequences are aligned based on the sequences in Figure 1. An example of this modified xylanase is identified as NI-BX2 in the Examples.

In a preferred embodiment, the selected xylanase for this modification consists of *Trichoderma reesei* xyn II, *Trichoderma harzianum* xyn, *Trichoderma viride* xyn, *Streptomyces lividans* xyn B,*Streptomyces lividans* xyn C, *Bacillus circulans* xyn A, *Bacillus subtilis* xyn A, *Aspergillus niger* xyn A, *Aspergillus kawachii* xyn A, or *Aspergillus tubigensis* xyn A.

Upstream extension consists of adding a sequence of up to 10 amino acids to the low molecular mass Family 11 xylanase upstream of the N-terminus. This upstream extension is carried out in combination with a chimeric modification of the selected Family 11 xylanase with *T. fusca* xylanase A described herein.

In a preferred embodiment, a tripeptide comprising glycine-arginine-(arginine or lysine) is added in the upstream extension from the N-terminus of the Family 11 xylanase, and this extension is carried out in combination with a chimeric modification. Examples of modified xylanases with these modifications are NI-TX9 and NI-BX7.

In a preferred embodiment, one to three basic amino acids are added between the N-terminus and a sequence of 5 to 9 amino acids from the Family 11 xylanase *Clostridium acetoburylicum* xynB (CaX), and this extension is carried out in combination with a chimeric modification. Examples of modified xylanases with these modifications are NI-TX8, NI-BX5, and NI-BX6 identified in the Examples.

In a preferred embodiment, a sequence of amino acids ASAR or ASAK are added upstream of the N-terminus and this extension is carried out in combination with a chimeric modification. Examples of modified xylanases with these modifications are NI-TX7, NI-BX3, and NI-BX4.

Another set of preferred embodiments involves using the modified enzymes of the present invention to treat pulp

and improve its bleachability. Said enzyme treatment is carried out at temperatures of 55 °C to 75 °C that are not in the acceptable range for enhancing the bleaching of pulp using natural Family 11 xylanases. In a preferred embodiment, said enzyme treatment is carried out at a pH of 7.5 to 9.0 that is not in the acceptable range for enhancing the bleaching of pulp using natural Family 11 xylanases.

It is well known to those skilled in the art that the xylanase enzymes of the invention might be useful in applications outside of pulp bleaching. For example, these enzymes might be useful as animal feed additives to aid in the digestibility of feedstuffs, where high temperature pelleting makes current enzymes unsuited in many cases. In addition, these enzymes might be useful in the processing of wheat and corn for starch production, in which the high process temperatures destroy current enzymes. It is well known to those skilled in the art that in these or other applications, the modified xylanase enzymes of the invention can be used in the presence of other enzymes, including but not limited to cellulase, mannanase, beta-glucanase, and amylase

The present invention will be further illustrated by detailed description of the following examples, which are not to be construed as limiting. Modified xylanases according to the Examples are listed in TABLES 2 and 3.

TABLE 2

Trichoderma reesei xylanases	
xylanase	description
natl TrX	natural *T. reesei* xylanase.
rec TrX	recombinant TrX produced by *E. coli*, without posttranslational modification.
TvX(3-190)	recombinant TrX with 5 different residues: Ala-1, Ser-2, Gly-4, Phe-9, Thr-65 and Thr-143.
NI-TX1	TvX(3-190) with a mutation Q162H.
NI-TX2	chimeric; the (1-29) sequence of NI-TX1 was substituted by the same region of Tfx and the N-terminus was extended upstream with the tetrapeptide ASHA added to positions (-4) to (-1).
NI-TX3	chimeric; the (1-29) sequence of NI-TX1 was substituted by the same region of Tfx.
NI-TX4	chimeric; the (10-29) sequence of NI-TX1 was substituted by the same region of Tfx.
NI-TX5	chimeric; the TYTN aa sequence in the (26-29) sequence of NI-TX1 was substituted by the same region of Tfx.
NI-TX6	NI-TX1 with mutations Y27M and N29L.
NI-TX7	chimeric; NI-TX3 was extended with the tetrapeptide ASAR in the (-4) to (-1) positions
NI-TX8	chimeric; NI-TX3 was extended upstream in the (-10) to (-1) positions, with (i) the *C. acetobutylicum* xynB (23-31) sequence from the (-10) to (-2) positions, and (ii) Arg at the (-1) position (Tfx aa numbering).
NI-TX9	chimeric; NI-TX3 was extended upstream with a tripeptide GRR from the (-3) to (-1) positions (Tfx aa numbering).
NI-TX10	NI-TX1 with mutations N10H and N11D.
NI-TX11	NI-TX1 with mutations N10H, Y27M, and N29L.
NI-TX12	NI-TX1 with mutations N10H, N11D, Y27M, and N29L.
NI-TX13	recombinant TrX with mutations N10H, Y27M, and N29L.

TABLE 3

Bacillus circulans xylanases	
xylanase	description
BcX	wild type *B. circulans* xylanase.
NI-BX1	chimeric; the (1-22) sequence of BcX was substituted by Tfx(1-31) sequence, and the N-terminus was extended with the tetrapeptide ASHA in the -1 to -4 positions. Modification is same as in NI-TX2.
NI-BX2	chimeric; the (1-22) sequence of BcX was substituted by Tfx(1-31) sequence. Modification is same as in NI-TX3.
NI-BX3	chimeric; N-terminus is identical to NI-BX2, but with the N-terminus extended with the tetrapeptide ASAR at the (-4) to (-1) position (Tfx aa numbering). Modification is same as in NI-TX7.
NI-BX4	chimeric; N-terminus is identical to NI-BX2, but with the tetrapeptide ASAK at the (-4) to (-1) positions (Tfx aa numbering).
NI-BX5	chimeric; the N-terminus extension of NI-BX2 was with (i) the *C. acetobutylicum* xynB (23-31) sequence at the (-10) to (-2) position (TfX aa numbering), and (ii) Arg at the (-1) position. Modification is same as in NI-TX8.
NI-BX6	chimeric; NI-BX5 with substitution by Arg at the (-2) position and Gly at the (-3) position (Tfx aa numbering).
NI-BX7	chimeric; NI-BX2 was extended upstream with a tripeptide GRR from the (-3) to (-1) positions (Tfx numbering). Modification is same as in NI-TX9.

Examples 1 through 3 hereafter will describe the production and purification of modified xylanases according to the invention. The suprisingly enhanced thermophilicity, alkalophilicity, and thermostability of modified xylanases NI-TX1 through NI-TX11 and NI-BX1 through NI-BX7 are demonstrated using xylan as the substrate in Examples 4 through 13. The performance of xylanase on a xylan substrate is well known to correlate well with that performance which is observed during actual treatment of pulp. A confirmation of suprisingly enhanced performance using modified xylanases according to the invention in the treatment of pulp, before bleaching, is provided for selected modified xylanases in Examples 14 and 15.

EXAMPLE 1

CONSTRUCTION OF THE TRICHODERMA REESEI MODIFIED XYLANASES NI-TX

Basic recombinant DNA methods like plasmid preparation, restriction enzyme digestion, polymerase chain reaction, oligonucleotide phosphorylation, ligation, transformation and DNA hybridization were performed according to well-established protocols familiar to those skilled in the art (Sung et al., 1986) or as recommended by the manufacturer of the enzymes or kit. The buffer for many enzymes have been supplied as part of a kit or reconstituted following the instruction of the manufacturers. Restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were purchased from New England BioLabs LTD, Mississauga, Ont. GeneAmp PCR reagent kit was purchased from Perkin-Elmer. A precursor plasmid pXYbc, which is a pUC type plasmid with a *Bacillus circulans* xylanase gene inserted, has previously been prepared and published (Sung et al, 1993; Campbell et a. US patent no. 5,405,769 issued on Apr. 11,1995). A commonly used *E. coli* strain, HB101 (Clonetech Lab, Palo Alto, CA) was used as transformation and expression host for all gene construct. Birchwood xylan was purchased from Sigma (St. Louis, Mo). Hydroxybenzoic acid hydrazide (HBAH) was purchased from Aldricht. Oligonucleotides were prepared with an Applied Biosystem DNA synthesizer, model 380B. Xylanase assays have been performed in a covered circulating water bath (Haake type F 4391) with a fluctuation of ±0.1°C. Temperature of the water bath was confirmed with a thermocouple.

A. Construction of the precursor plasmid pTvX(3-190).

The precursor plasmid pTvX(3-190) for all subsequent mutations has previously prepared and its construction has already been published (Sung et al, 1995). This plasmid is derived from plasmid pXYbc, through a substitution of the *Bacillus circulans* xylanase gene of the latter by a newly assembled *Trichoderma* xylanase gene. Expression of this

Trichoderma xylanase and other mutant xylanases subsequently described are under the control of the *lac* promoter. The total assembly of the *Trichoderma* xylanase gene required two stages, initially for the (92-190) region, then followed by the (1-92) region. The protocol for the construction of this gene is routine and identical to the standard published procedure for many other genes, using enzymatic phosphorylation of overlapping synthetic oligonucleotides which encodes xylanase, followed by their ligation into an appropriately cut plasmid.

Initially ten overlapping oligonucleotides (XyTv-101 to 110) encoding the TvX(92-190) sequence (Figure 3), were designed with codon usage frequency imitating that of *E. coli*. The SalI and BglII cohesive ends of two terminal oligonucleotides enabled the enzymatic ligation of the ten fragments to the SalI-BglII linearized plasmid pXYbc. Ten oligonucleotides XyTv-101 to XyTv-110 (50 pmol, 1 μL for each) encoding the (92-190) region of *Trichoderma* xylanase was phosphorylated in a mixture containing 10X standard kinase buffer (0.4 μL), 1mM ATP (4 μL), T4 DNA kinase (5 units), and water (3 μL). Phosphorylation reaction was carried out for 1 h at 37°C. The solutions were then combined and heated to 70°C for 10 min. After being cooled slowly to room temperature, the combined solutions were added to a mixture of 4mM ATP (3.5 μL), SalI-BglII linearized plasmid pXYbc (0.1 pmol), and T4 DNA ligase (3.5 μL) and incubated at 12°C for 20 h. Aliquots of the ligation mixture were used to transform *E. coli* HB101 in YT plate (8 g yeast extract, 5 g bacto-tryptone, 5 g NaCl, 15 g of agar in 1 L of water) containing ampicillin (100 mg/L).

For the preparation of a hybridization probe, one of the oligonucleotide XyTv-110 (10 pmol, 1 μL) was phosphorylated ^{32}P-ATP (10 pmol, 3 μL) in T4 DNA kinase (1 μL), 10X kinase buffer (1 μL), and water (4 μL) at 37°C for 1 h.

Transformants were selected randomly for hybridization analysis. Colonies were grown on nylon filters on YT plates with ampicillin overnight. They were then denatured with 0.5N NaOH - 1.5M NaCl (10 min) and neutralized with 0.5N Tris-HCl (pH 7.0) - 1.5M NaCl (10 min). After irradiation by UV of 254 nm for 8 min, the filters were washed with 6X SSC - 0.05% TritonX-100 for 30 min. Cell debris was scraped off completely. After another 30 min. in fresh solution, the duplicate filters were transferred individually into separate mixtures of 6X SSC - 1% dextran sulphate - 0.05% TritonX-100 - 1X Denhardt's hybridization fluid. The ^{32}P-labelled probe was added to the filter. After 16 h at 45°C, the filter was washed twice with 6X SSC - 0.05% TritonX-100 at room temperature for 5 min. and then at 65°C for 30 min. Positively hybridized clones with the intermediate plasmid pBcX.TvX were identified by auto-radiographic analysis.

The above protocol, involving enzymatic phosphorylation of synthetic overlapping oligonucleotides and ligation into a linearized plasmid, has again been used in the assembly of the TX(1-92) region and in the cassette mutagenesis for the subsequent generation of several mutant series NI-TX and NI-BX described in this invention.

For the assembly of the TX(1-92) region to complete the full-length *Trichoderma* gene, the intermediate plasmid pBcX.TvX was linearized by NheI and KpnI endonucleases to release the DNA insert for BcX(1-83). With NheI and KpnI cohesive ends, ten overlapping oligonucleotides (XyTv-1 to -10) encoding the published TvX(3-91) sequence wer ligated into the linearized plasmid pBcX.TvX (Figure 3), via the protocol described above. The new plasmid pTvX(3-190) therefore harbored a synthetic TvX(3-190) gene. As compared to the natural TrX, the recombinant TvX(3-190) has five different residues: Ala-1, Ser-2, Gly-4, Phe-9, Thr-65 and Thr-143.

For comparison, a gene encoding the natural TrX has also been assembled in the same manner with the five natural residues. This has also been published (Sung et al, 1995). Expression of this gene in *E. coli* generated a recombinant version of TrX (rec. TrX). As indicated below and in the same report by Sung et al (1995), the performance characteristics of both TvX(3-190) and rec. TrX were identical. However, both *E. coli*-expressed xylanases were worse than the natural TrX (natl TrX) in thermophilicity and thermostability, probably due to the lack of posttranslational modification as in the case of natural TrX.

B. Construction of the plasmid pNI-TX1

All mutant xylanases genes including both NI-TX and NI-BX series described below have been constructed via the method of cassette mutagenesis. The protocol for the cassette mutagenesis was identical to that for gene assembly fully described above. Such cassette mutagenesis involved (i) enzymatic phosphorylation of overlapping synthetic oligonucleotides, (ii) their ligation with the linearized plasmid, (iii) transformation into the *E. coli* HB101 competent cells, (iv) identification of the mutant transformants via hybridization with the labelled oligonucleotide as probe, and (v) confirmation of the mutation through dideoxy nucleotide sequencing.

The mutant NI-TX1 was identical to TvX(3-190) with a single mutation Q162H. The construction of the plasmid pNI-TX1 was through ligation of oligonucleotides TX-162H-1 and TX-162H-2 (shown below) into NsiI/AvrII-linearized plasmid pTvX(3-190) in a cassette mutagenesis.

```
                    TX-162H-1
158 159 160 161 162 163 164 165 166
      A   W   A   Q   H   G   L   T   L
    5'-TGG GCA CAG CAC GGG TTA ACC
A CGT ACC CGT GTC GTG CCC AAT TGG GAT C-5'
NsiI                          AvrII
                    TX-162H-2
```

C. Construction of the plasmid pNI-TX2

The mutant NI-TX2 is a modified version of NI-TX1, with (i) the (1-29) sequence of the latter substituted by the Tfx(1-29) sequence and (ii) the N-terminus extended upstream with the tetrapeptide ASHA in positions -4 to -1. The plasmid pNI-TX2 was constructed through the ligation of overlapping oligonucleotides Tfx-1, -2, -3 and -4, that encoded the Tfx(1-29) sequence, to the NheI/ApaI-linearized plasmid of pNI-TX1 in a cassette mutagenesis.

```
                                                    Tfx-1
                          Tfx
          -4  -3  -2  -1   1   2   3   4   5   6   7   8   9  10
11  12
           A   S   H   A   A   V   T   S   N   E   T   G   Y   H
D   G
     5'-CT AGC CAC GCG GCC GTA ACT TCA AAT GAA ACC GGT TAT CAT
GAC GGC      _____G GTG CGC CGG CAT TGA AGT TTA CTT TGG CCA ATA GTA
CTG CCG   NheI                              Tfx-3          PinAI

                                                              Tfx-2
          13  14  15  16  17  18  19  20  21  22  23  24  25  26
27  28
           Y   F   Y   S   F   W   T   D   A   P   G   T   V   S
M   E
        TAT TTC TAC AGC TTC TGG ACC GAT GCA CCG GGA ACT GTG TCC
ATG GAG
        ATA AAG ATG TCG AAG ACC TGG CTA CGT GGC CCT TGA CAC AGG
TAC CTC
                                                            Tfx-4

          29  30  31
           L   G   P
        CTC  GGG  CC
        GAG C_____
             ApaI
```

D. Construction of the plasmid pNI-TX3

The mutant NI-TX3 is a modified version of NI-TX2, without the extra tetrapeptide ASHA from the (-4) to (-1) positions as in the latter. The plasmid pNI-TX3 was prepared via substitution of the coding sequence for Tfx(1-6) in the NI-TX2 gene with a new coding sequence for TrX (1-6) minus the extra upstream residues. This was accomplished via ligation of the oligonucleotides Tfx(1-6)-1 and Tfx(1-6)-2 into the NheI/PinAI-linearized plasmid pNI-TX2 in a cassette mutagenesis.

```
                                        Tfx(1-6)-1
                                Tfx  1   2   3   4   5   6   7   8
                               fmet  A   V   T   S   N   E   T   G
5-CT AGC TAA GGA GG CTG CAG ATG GCA GTA ACA TCA AAT GAA A
     G ATT CCT CC GAC GTC TAC CGT CAT TGT AGT TTA CTT TGG CC
  NheI                                Tfx(1-6)-2              PinAI
```

E. Construction of the plasmid pNI-TX4

The mutant NI-TX4 is a modified version of NI-TX1, with the (10-29) sequence of the latter substituted by the Tfx(10-29) sequence. The plasmid pNI-TX4 was prepared via substitution of the coding sequence for Tfx(1-6) in NI-TX2 gene with that of TrX (1-6). This was accomplished via ligation of the oligonucleotides TX-1f and TX-8f into the NheI/PinAI-linearized plasmid pNI-TX2 in a cassette mutagenesis.

```
                                          TX-1f
                                  Tx 1   2   3   4   5   6   7
                                 st  Q   T   I   Q   P   G   T
5'-CT AGC TAA GGA GG CTG CAG ATG CAA ACA ATA CAA CCA GGA A
   3'-G ATT CCT CC GAC GTC TAC GTT TGT TAT GTT GGT CCT TGG CC
    NheI                          TX-8f                  PinAI
```

F. Construction of the plasmids pNI-TX5 and pTX-28E-29L-162H

The mutant NI-TX5 was identical to NI-TX1, but with the tetrapeptide TYTN aa sequence in the (26-29) region of the latter substituted by the corresponding tetrapeptide SMEL in the (26-29) region of Tfx. The construction of the plasmid pNI-TX5 was through ligation of the heteroduplex oligonucleotides TX-26SMEL-1 and TX-28E/29L-1 (shown below) into NcoI/ApaI-linearized plasmid pNI-TX1 in a the cassette mutagenesis. Subcloning of the plasmid preparation yielded the two target plasmids.

```
                        TX-26SMEL-1
      22  23  24  25  26  27  28  29  30  31
       H   G   G   V   S   M   E   L   G   P
5'-CAT GGT GGT GTG AGC ATG GAG CTC GGG CC
    CA CCA CAC TGG ATG CTC GAG C-5'
  NcoI              T   Y          ApaI
                    TX-28E/29L-1
```

As the two oligonucleotides are heteroduplex, subcloning and hybridization with TX-26SMEL-1 and TX-28E/29L-1 yielded two plasmids, (i) pNI-TX5 with SMEL at the (26-29) region, and (ii) pTX-28E-29L-162H with E and L at positions- 28 and 29.

G. Construction of the plasmid pNI-TX6

The mutant NI-TX6 was identical to NI-TX1, but with two single mutations Y27M and N29L. The construction of the plasmid pNI-TX6 was through ligation of oligonucleotides TX-27M/29L-1 and TX-27M/29L-2 (shown below) into

NcoI/ApaI-linearized plasmid pNI-TX1 in a cassette mutagenesis.

```
                    TX-27M/29L-1
      22  23  24  25  26  27  28  29  30  31
       H   G   G   V   T   M   T   L   G   P
5'-CAT GGA GGC GTC ACA ATG ACT CTG GGG CC
    _____CT CCG CAG TGT TAC TGA GAC C
     NcoI                              ApaI
                    TX-27M/29L-2
```

H. Construction of the plasmid pTX-27M-162H

The mutant TX-27M-162H was identical to NI-TX1, but with an additional mutation Y27M. The construction of the plasmid was through ligation of duplex oligonucleotides TX-27M-1 and TX-27M-2 (shown below) into the NcoI/ApaI-linearized plasmid pNI-TX1 in a cassette mutagenesis.

```
                    TX-27M-1
      22  23  24  25  26  27  28  29  30  31
       H   G   G   V   T   M   T   N   G   P
5'-CAT GGA GGC GTC ACA ATG ACT AAT GGG CC
    _____CT CCG CAG TGT TAC TGA TTA C_____
     NcoI                              ApaI
                    TX-27M-2
```

I. Construction of the plasmid pNI-TX7

The mutant NI-TX7 is a modified version of NI-TX3, with the tetrapeptide ASAR in the -4 to -1 positions. The construction of the plasmid pNI-TX7 was accomplished via ligation of the oligonucleotides Tf-(-1)R-1 and Tf-(-1)R-2 into the NheI/PinAI-linearized plasmid pNI-TX2 in a cassette mutagenesis.

```
                              Tf-(-1)R-1
                         Tfx
                   -1   1   2   3   4   5   6   7   8
    A   S   A   R   A   V   T   S   N   E   T   G
5'-CT AGC GCA AGA GCA GTA ACA AGT AAC GAG A
    _____G CGT TCT CGT CAT TGT TCA TTG CTC TGG CC
     NheI                                   PinAI
                              Tf-(-1)R-2
```

J. Construction of the plasmid pNI-TX8

The mutant NI-TX8 is a modified version of NI-TX3, with the N-terminus of the latter extended upstream (i) with *C. acetobutylicum* xynB (23-31) sequence from (-10) to (-2) positions, and (ii) an Arg residue at (-1) position (Tfx aa numbering). The construction of the plasmid pNI-TX8 was accomplished via ligation of the oligonucleotides CaTf-(-1)R-1 and CaTf-(-1)R-2 into the NheI/PinAI-linearized plasmid pNI-TX2 in a cassette mutagenesis.

```
                                        CaTf-(-1)R-1
      CaX                                          Tfx
        -10  -9   -8   -7   -6   -5   -4   -3   -2   -1    1    2    3    4    5
    A    S    A    F    N    T    Q    A    A    P    R    A    V    T    S    N
5'CT AGC GCA TTC AAC ACA CAG GCC GCT CCT CGA GCT GTC ACC AGC AAC
     _____G CGT AAG TTG TGT GTC CGG CGA GGA GCT CGA CAG TGG TCG TTG
      NheI                          CaTf-(-1)R2
```

```
      6    7    8
      E    T    G
   GAG A
   CTC TGG CC
       PinAI
```

K. Construction of the plasmid pNI-TX9

The mutant NI-TX9 is a modified version of NI-TX3, with the N-terminus extended upstream with a tripeptide GRR from the (-3) to (-1) positions (Tfx aa numbering).

PCR primers were constructed to introduce the tripeptide GRR from the (-3) to (-1) positions. The 5' and 3' primers are shown below:

```
                                  5' primer, GRR-Tf(1-6)-1
                                        TfX
                          -3   -2   -1    1    2    3    4    5    6    7    8
                      st   G    R    R    A    V    T    S    N    E    T    G
5' ACT CTG CAG ATG GGA AGA AGG GCC GTA ACT TCA AAT GAA ACC GGT
       PstI
```

```
     9   10   11
     Y    H    D
   TAT CAT GAC
```

```
                                  3' primer, Uni-PCR-1r

                5' GAA AAG TGC CAC CTG ACG TCC CAA GCT T
                                                HindIII
```

The plasmid pNI-TX2 was used as a template for PCR. The reaction solution contained the plasmid pNI-TX2 DNA (50 ng, 15 µL), 5 µL of 10X buffer (100mM KCl, 100mM ammonium sulfate, 200mM Tris-HCl pH 8.8, 40 mM magnesium sulfate, 1% TritonX-100, 100 µg/ml BSA), 5 µL of 5mM dNTPs, 5' primer solution (25 pmol, 2.5 µL), 3' primer solution (25 pmol, 2.5 µL) and water (19 µL).

The reaction was covered with paraffin oil (50 µL) to prevent evaporation. The reaction mixture was pre-warmed to 94°C without enzyme for 5 min., then the reaction mixture was cooled to 72°C, then the enzyme DNA polymerase (1 µL,1U) was added. The reaction was incubated in a temperature cycler for 30 cycles of 94°C for 1 min., 55°C for 2 min. and then 72°C for 2 min. The yield of the PCR product was approximately 1 µg of a 600 bp fragment. This fragment was purified from an agarose gel.

The resulting PCR product was cut with restriction enzymes PstI and HindIII, and was subsequently ligated into the PstI/HindIII-linearized plasmid pNI-TX3 in a cassette mutagenesis. Transformation in *E. coli* HB101 yielded the plasmid

pNI-TX9.

L. Construction of the plasmid pNI-TX10

The mutant NI-TX10 was identical to NI-TX1, but with two single mutations H10N and D11N. The construction of the plasmid pNI-TX12 was through ligation of oligonucleotides TX10HD-1 and TX10HD-2 (shown below) into the PinAI/ApaI-linearized plasmid pNI-TX1 in a cassette mutagenesis.

```
                                          TX10HD-2
   7   8   9  10  11  12  13  14  15  16  17  18  19  20  21  22
   T   G   F   H   D   G   Y   F   Y   S   Y   W   N   D   G   H
5'CC GGT TTC CAC GAC GGT TAC TTT TAC AGC TAT TGG AAC GAC GGC CAT
   _____A AAG GTG CTG CCA ATG AAA ATG TCG ATA ACC TTG CTG CCG GTA
  PinAI                              TX10HD-1

  23  24  25  26  27  28  29  30  31
   G   G   V   T   Y   T   N   G   P
 GGA GGA GTA ACT TAC ACC AAT GGG CC
 CCT CCT CAT TGA ATG TGG TTA C_____
                             ApaI
```

M. Construction of the plasmid pNI-TX11 and pNI-TX12

The mutant NI-TX11 and NI-TX12 were identical to NI-TX1 with three and four single mutations respectively. NI-TX11 has three mutations N10H, Y27M and N29L. NI-TX12 has four mutations N10H, N11D, Y27M and N29L.

The desirable gene has been constructed through PCR with the plasmid pNI-TX6 a template. The 5' and 3' PCR primers TX10HD/N-1 and Uni-PCR-1r, and the PCR protocol for the introduction of the tripeptide GRR have been described in the construction of pNI-TX9.

The resulting PCR product was cut with restriction enzymes PinAI and HindIII, and was subsequently ligated into the PinAI/HindIII-linearized plasmid pNI-TX1 in a cassette mutagenesis. Tranformation in *E. coli* HB101 yielded both the plasmids pNI-11 and pNI-12. Their identities were established by nucleotide sequencing.

```
                5'primer                TX10HD/N-1
          7   8   9   10  11  12  13  14  15  16  17  18  19  20
          T   G   Y   H  D/N  G   Y     F  Y   S   Y   W   N   D
5'GAA ACC GGT TAC CAC XAC GGT TAC TTT TAC AGC TAT TGG AAC GAT
      Pin AI

  21  22
   G   H
 GGC C

 X = G + A
```

N. Construction of the plasmid pNI-TX13

The mutant NI-TX13, is identical to the recombinant wild type TrX, but with the three mutations N10H, Y27M and N29L described in NI-TX11. The generation of this mutant requires the conversion of the residues at positions 1-4 in mutant NI-TX11 to those of TrX. Three residues Ala-1, Ser-2, Gly-4 in mutant NI-TX11 has been converted into Gln, Thr and Gln respectively. The construction of the plasmid pNI-TX13 was through ligation of oligonucleotides TrX1f and TrX8f

(shown below) into the NheI/PinAI-linearized plasmid pNI-TX11 in a cassette mutagenesis.

```
                                                TrX1f
                                        TrX   1   2   3   4   5   6   7   8
                                       fmet   Q   T   I   Q   P   G   T   G
5-CT AGC TAA GGA GG CTG CAG ATG CAA ACA ATA CAA CCA GGA A
   _____G ATT CCT CC GAC GTC TAC GTT TGT TAT GTT GGT CCT TGG CC
    NheI                                TrX8f                    PinAI
```

EXAMPLE 2

CONSTRUCTION OF THE *BACILLUS CIRCULANS* MUTANT XYLANASES NI-BX

The modifications in the fungal *Trichoderma* xylanases NI-TX2, NI-TX3, NI-TX7, NI-TX8 and NI-TX9 have also been repeated in the bacterial *B. circulans* xylanase (BcX). The precursor plasmid pXYbc has previously prepared and published (Sung et al, 1993; Campbell et a. US patent no. 5,405,769 issued on Apr. 11, 1995), and would only be briefly described. A synthetic gene encoding the *B. circulans* xylanase (BcX) was assembled through the enzymatic phosphorylation by T4 DNA kinase and ligation of overlapping synthetic oligonucleotides into a linearized plasmid by T4 DNA ligase (FIG. 4)(Sung et al, 1993), via the same protocol described above for pXyTv(3-190).

A. Construction of the plasmid pNI-BX1

The modification in NI-BX1 has been applied in the preparation of NI-TX2. The mutant NI-BX1 is a modified version of BcX, with a substitution of the (1-22) region of the latter by Tfx(1-31) sequence. The plasmid pNI-BX1 was constructed through the ligation of overlapping oligonucleotides Tfx-1, -2b, -3 and -4b, that encoded the Tfx(1-31) sequence, to the NheI/BspEI-linearized plasmid of pXYBc in a cassette mutagenesis.

```
                                                Tfx-2b
  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25  26
   D   G   Y   F   Y   S   F   W   T   D   A   P   G   T   V   S
                                  ACC GAT GCC CCG  GGA ACT  GTG AGT
3'-G CCG ATA AAG ATG TCG AAG ACC TGG CTA CGG  GGC CCT TGA  CAC TCA
                                  Tfx-4b

 27  28  29  30  31
  M   E   L   G   P
ATG GAG CTC GGC C
TAC CTC GAG CCG GGG CC
                  BspEI
```

B. Construction of the plasmid pNI-BX2

This modification in NI-BX2 has been applied in the preparation of NI-TX3. The mutant NI-BX2 is a modified version of NI-BX1, but without the extra residues from the (-4) to (-1) positions as in the latter. The plasmid pNI-BX2 was prepared via substitution of the coding sequence for Tfx(1-6) in the NI-BX1 gene with a new coding sequence for TrX(1-6) minus the upstream extra residues. This was accomplished via ligation of the oligonucleotides Tfx(1-6)-1 and Tfx(1-6)-2 into the NheI/PinAI-linearized plasmid pNI-BX1 in a cassette mutagenesis.

C. Construction of the plasmid pNI-BX3

The modification in NI-BX3 has been applied in the preparation of NI-TX7. The mutant NI-BX3 is a modified version of NI-BX1, but with its N-terminus extended by one Arg residue at the (-1) position. The construction of the plasmid pNI-BX3 was accomplished via ligation of the oligonucleotides Tf-(-1)R-1 and Tf-(-1)R-2 into the NheI/PinAI-linearized plasmid pNI-BX1 in a cassette mutagenesis.

D. Construction of the plasmid pNI-BX4

The mutant NI-BX4 is identical to NI-BX3, but with an Arg residue replaced by a Lys residue at the (-1) position. The construction of the plasmid pNI-BX4 was accomplished via ligation of the oligonucleotides Tf-(-1)K-1 and Tf-(-1)K-2 into the NheI/PinAI-linearized plasmid pNI-BX1 in a cassette mutagenesis.

```
                                        Tf-(-1)K-1
                                    Tfx
                      -1   1   2   3   4   5   6   7   8
    A   S   A   K   A   V   T   S   N   E   T   G
5'-CT AGC GCA AAA GCA GTA ACA AGT AAC GAG A
   -    G CGT TTT CGT CAT TGT TCA TTG CTC TGG CC
    NheI                                        PinAI
                                    Tf-(-1)K-2
```

E. Construction of the plasmids pNI-BX4-K(-1)D and pNI-BX4-K(-1)E

The mutant NI-BX4-K(-1)D and NI-BX4-K(-1)E are identical to NI-BX4 or NI-BX3, but with an acidic residue Asp or Glu substituting the basic residue at the (-1) position. The construction of the plasmids was accomplished via ligation of the heteroduplex oligonucleotides Tf-(-1)D-1 and Tf-(-1)E-2 into the NheI/PinAI-linearized plasmid pNI-BX1 in a cassette mutagenesis. Subcloning of the prepared plasmid yielded the target plasmids.

```
                                    Tf-(-1)D-1
                                Tfx
                      -1   1   2   3   4   5   6   7   8
    A   S   A   D   A   V   T   S   N   E   T   G
5'-CT AGC GCA GAT GCA GTA ACA AGT AAC GAG A
    _   G CGT CTT CGT CAT TGT TCA TTG CTC TGG CC
    NheI        E                           PinAI
                                    Tf-(-1)E-2
```

F. Construction of the plasmid pNI-BX5

The modification in NI-BX5 has been applied in the preparation of NI-TX8. The mutant NI-BX5 has (i) a substitution of its (1-29) sequence by the Tfx(1-29) aa sequence, (ii) an extension of an Arg residue at (-1) position (Tfx aa numbering), and (iii) a further extension upstream with an *C. acetobutylicum* xynB (23-31) sequence from (-10) to (-2) positions. The construction of the plasmid pNI-BX5 was accomplished via ligation of the oligonucleotides CaTf-(-1)R-1 and CaTf-(-1)R-2 into the NheI/PinAI-linearized plasmid pNI-BX1 in a cassette mutagenesis.

G. Construction of the plasmid pNI-BX6

PCR primers were constructed to generate variation at the positions-(-3) and (-2) of NI-BX5. Mixed bases encoded residues Ser and Gly at position-(-3), and Pro, Ala, Gly and Arg at position-(-2). The 5' and 3' primers are shown below:

```
                                  5' primer, CaTf-PCR-1
                    CaX                                -3   -2        Tfx
                    -10   -9   -8   -7   -6   -5   -4       P/A   -1    1    2    3
    4
           A    S    A    F    N    T    Q    A S/G G/R    R    A    V    T    S
5'CCC GCT AGC GCA TTC AAC ACA CAA GCA XGT YYA AGG GCC GTA ACT TCA
          NheI

  5    6    7    8    9
  N    E    T    G    Y
AAT GAA ACC GGT T

where X = A and G mixture
          Y = G and C mixture


                                  3' primer, Xy-14a
  BcX
   49   50   51   52   53   54   55   56   57   58   59   60   61
3'GCA TGC TAG TTG ATG TTG CGA CCG CAA ACC CGG GGC TTA-5'
```

The plasmid pNI-BX1 was used as a template for PCR. The PCR protocol has been described in the construction of NI-TX9. The yield of the PCR product was approximately 1 μg of a 200 bp fragment. This fragment was purified from an agarose gel.

The resulting PCR product was cut with restriction enzymes NheI and BamHI and was subsequently ligated into the NheI/BamHI-linearized plasmid pXYBc in a cassette mutagenesis. Tranformation in *E. coli* HB101 yielded plasmids harboring different xylanase genes encoding different residues at the (-3) and (-2) position. After the study of the enzymatic characteristics of the expressed xylanases in the Examples below, the most improved mutant was NI-BX6. Dideoxy nucleotide sequencing of NI-BX6 revealed residues Gly and Arg respectively in positions-(-3) and (-2).

H. Construction of the plasmid pNI-BX7

The mutant NI-BX7 is a modified version of NI-BX2, with the N-terminus extended upstream with a tripeptide GRR from the (-3) to (-1) positions (Tfx aa numbering).

The desirable gene has been constructed through PCR with the plasmid pNI-BX1 as a template. The PCR primers GRR-Tf(1-6)-1 and Uni-PCR-1r, and the PCR protocol for the introduction of the tripeptide GRR have been described in the construction of NI-TX9.

The resulting PCR product was cut with restriction enzymes PstI and HindIII, and was subsequently ligated into the PstI/HindIII-linearized plasmid pNI-TX3 in a cassette mutagenesis. Tranformation in *E. coli* HB101 yielded the plasmid pNI-BX7.

EXAMPLE 3

PRODUCTION AND ASSAYS OF MODIFIED XYLANASES

(A) Production of xylanases

The culture condition was identical to the well-established protocol described for other *E. coli*-expressed xylanases. A 5 ml of overnight innoculant in 2YT medium (16 g yeast extract, 10 g bacto-tryptone, 5 g NaCl, 1 L of water) containing ampicillin (100 mg/L) was added to 2YT medium (1 L) with ampicillin. The cultures were grown for with shaking (200 rpm) at 37°C. After 16 hr, cells were harvested.

(B) Purification of modified xylanases

Protein samples were prepared from cells by first making an extract of the cells by grinding 10 g of the cell paste with 25 g of alumina powder. After grinding to smooth mixture, small amounts (5 mL) of ice cold buffer A (10mM sodium acetate, pH 5.5 for BcX mutants) or buffer B (10mM sodium acetate, pH 4.6 for TX mutants) were added and the mixture ground vigorously between additions. The alumina and cell debris were removed by centrifugation of the mixture at 8000 x g for 30 min.

Before column chromatography, the supernatant (25 mL) of modified xylanases were pretreated in one of the following ways:

(1) NI-BX1, NI-BX2, NI-TX1, NI-TX2, NI-TX3, NI-TX4, NI-TX6,NI-TX7, NI-TX10, NI-TX11, NI-TX12, NI-TX13.
Dialysis overnight at 4°C against 3 L buffer A, with dialysis tubing (3500 molecular weight cutoff). A slight precipitate formed in the dialysis bag, which is removed by centrifugation at 8000xg for 15 min.

(2) NI-BX3, NI-BX4, NI-BX5, NI-BX6, NI-BX7
Heating at 60°C for 20 min, then 68°C for 30 min and centrifugation to remove a large amount of precipitate. The supernatant was acidified to pH 4.6, frozen at -20°C overnight thawed and centrifuged to remove more precipitate.

(3) NI-TX8, NI-TX9
Heating at 60°C for 30 min and centrifugation to remove a large amount of precipitate. The supernatent was acidified to pH 4.6, frozen at -20°C overnight, thawed and centrifuged to remove more precipitate.

(4) NI-TX5
Without dialysis or heating, the crude supernatent was directly acidified to pH 4.6, frozen at -20°C overnight, thawed and centrifuged to remove more precipitate.

After the above pretreatment, the cell extract was pumped onto a 50 mL bed volume, S-sepharose fast flow, cation exchange column (Kabi-Pharmacia, Canada), equilibrated in buffer A. The xylanase was eluted with a 300 mL linear gradient of 0 to 0.3M NaCl in buffer A at a flow rate of 3 mL/min. The xylanase elutes at 100 to 150 mL of the gradient. The fractions are checked on SDS-PAGE, and those fractions having most of the xylanase were pooled, and concentrated by ultrafiltration using 3000 dalton molecular weight cutoff membranes (Amicon YM3). The concentrated material (5 mL) was then applied to a 1.5 cm x 85 cm TSK-HW50S gel filtration column, equilibrated in 50 mM ammonium acetate pH 6. The xylanase eluted at a volume of 90 to 100 mL. These fractions were analyzed by SDS-PAGE, and the peaks pooled as pure xylanase. The protein was quantified using the extinction coefficient at 280 nm. Typical purified yield from 10 g of cells was 25 mg of xylanase. All NI-TX and NI-BX xylanases have good solubility in the ammonium acetate buffer without glycerol.

(C) Standard assay for the measurement of enzymatic activity

The quantitative assay determined the number of reducing sugar ends generated from soluble xylan. The substrate for this assay was the fraction of birchwood xylan which dissolved in water from a 5% suspension of birchwood xylan (Sigma Chemical Co.). After removing the insoluble fraction, the supernatant was freeze dried and stored in a dessicator. The measurement of specific activity was performed as follows. Reaction mixtures containing 100 µL of 30 mg/mL xylan previously diluted in assay buffer (50 mM sodium citrate, pH 5.5 or the pH optimum of the tested xylanase), 150 µL assay buffer, 50 µL of enzyme diluted in assay buffer were incubated at 40°C. At various time intervals 50 µL portions were removed and the reaction stopped by diluting in 1 mL of 5mM NaOH. The amount of reducing sugars was determined with the hydroxybenzoic acid hydrazide reagent (HBAH) (Lever, 1972, Analytical Biochem 47:273-279). A unit of enzyme activity was defined as that amount generating 1µ mol reducing sugar in 1 minute at 40°C.

For the comparison between modified and the natural xylanases (Tables 4 and 5), the specific activities of the xylanases was normalized to the specific activity of the natural xylanase.

TABLE 4

Relative activity of NI-TX xylanases	
Xylanase	Relative activity %
natl TrX	100*
TvX(3-190)	102
NI-TX1	98
NI-TX2	92
NI-TX6	95
NI-TX6	99
NI-TX7	90

* The data are normalized to the specific activity of the natural TrX of 770 U/mg.

TABLE 5

Relative activity of NI-BX xylanases	
Xylanase	Relative activity %
BcX	100*
NI-BX1	92
NI-BX3	89
NI-BX6	92

* The data are normalized to the specific activity of BcX of 330 U/mg.

In both NI-TX (Table 4) and NI-BX xylanases (Table 5), the specific enzymatic activities of the modified xylanases at 40°C are not significantly different from the natural xylanases.

EXAMPLE 4

TEMPERATURE/ACTIVITY PROFILES OF MODIFIED TRICHODERMA XYLANASES

This was a measure of the effect of temperature on the enzymatic activity of the xylanase in the hydrolysis of soluble xylan. The procedure was identical to the standard assay with changes in the incubation temperature and time. The enzymes (15 µg/mL) and soluble xylanase in 50 mM sodium citrate buffer of pH 5.5 were mixed and incubated in a circulating water bath at different temperatures. After 30 min, the amount of reducing sugars released from xylan was determined by HBAH and was calculated as a relative activity, with the value at 40°C as 100%.

The effect of temperature on the hydrolysis of xylan is shown in Figure 5. The *E. coli*-expressed TvX(3-190) showed much less activity at 55°C or above than the natural TrX. As reported by Sung et al 1995, similarly low thermophilicity has been shown in the *E. coli*-expressed TrX.

The natural TrX was highly active up to 60°C. The advantage in thermotolerance of this enzyme over that expressed in *E. coli* is probably the result of posttranslational modifications in the *Trichoderma* host.

The mutant NI-TX1, with mutation Q162H, has an activity/temperature profile identical to that of TvX(3-190).

Substitution of the (1-29) region of NI-TX1 with the corresponding sequence of Tfx(1-29) and the N-terminus extension with the tetrapeptide ASHA at the (-4) to (-1) positions generated NI-TX2. If the effective temperature is defined as a temperature allowing 150% relative activity as at 40 °C, this mutant showed a gain of 10°C over TvX(3-190) or NI-

TX1.

A mutant xylanase NI-TX3 with the substitution by Tfx (1-29) but without the N-terminus extension of residues ASHA at the (-4) to the (-1) positions as in NI-TX2, has a temperature/activity profile identical to the latter.

Another mutant NI-TX4 with a substitution of a shorter Tfx(10-29) sequence has a slightly lower thermophilicity than NI-TX2 and NI-TX3.

The chimeric xylanase NI-TX5, with a change of the tetrapeptide from Tfx at the (26-29) region showed a gain of 5 °C over TvX (3-190) or NI-TX1.

The mutant NI-TX6, with double mutations Y27M and N29L retained significant enzymatic activity up to 60°C. It showed the same temperature/activity profile as NI-TX5.

Another mutant NI-TX7 has the same thermophilicity as NI-TX2. NI-TX7 has the same amino acid sequence as NI-TX3 but with the N-terminus extension consisting of a tetrapeptide ASAR in the -4 to -1 positions. Although this mutant and NI-TX2 have shown that extension with these tetrapeptides has no significant effect on the temperature range of the chimeric *Trichoderma* xylanase, other N-terminal extensions have been explored.

NI-TX8 was synthesized and it showed a gain of 13°C over TvX(3-190) or NI-TX1, and about 10°C over natural TrX. It is of the same structure as NI-TX7 with an additional extension upstream of the N-terminus with the *C. acetobutylicum* xynB (23-31) sequence from the (-10) to (-2) positions.

A mutant NI-TX9 was constructed to test whether the *C. acetobutylicum* xynB (23-31) sequence in upstream extension of NI-TX8 can be replaced by a shorter tripeptide sequence GRR. This tripeptide has been identified in the study of NI-BX7 described later. The new mutant NI-TX9 of smaller size showed the same temperature/activity profile as NI-TX8.

This is the first report of enhancement of performance by an upstream extension to the N-terminus of a xylanase.

As for the identification of other residues contributing to the thermophilicity of the Family 11 xylanase, a mutant NI-TX10 was prepared, which was derived from NI-TX1 with two mutations N10H and N11D. The new mutant NI-TX10 showed a gain of 6°C in its effective temperature as compared to its precursor NI-TX1.

Finally two mutant xylanases NI-TX11 and NI-TX12, derivatives of NI-TX1 with the triple mutation (N10H Y27M N29L) and the quadruple mutation (N10H N11D Y27M N29L) respectively, have been constructed. They were used to determine (i) the individual contribution of the mutations N10H and N11D, and (ii) their combined effect with two other advantageous mutations Y27M and N29L identified above. Both mutants NI-TX11 and NI-TX12 have shown identical temperature-activity profile with a gain of 13°C in effective temperature. This result indicated that the N11D mutation has no effect on the thermophilicity of TrX. A conversion of the residues at position (1-4) in NI-TX11 to those of the wild type TrX yielded NI-TX13. The temperature-activity profile of both NI-TX11 and NI-TX13 remained identical.

This gain in the effective temperature for a relative activity of 150% by NI-TX11 or NI-TX13 (+13°C) is greater than the theoretical sum of the gains by individual mutations N10H (+6°C, in NI-TX11) and Y27M/N29L (+4°C, in NI-TX2). This improvement is also greater than any gain via a direct substitution with any natural TfX sequence which may harbor the triple mutation, as shown in the chimeric mutants NI-TX2 (+10°C), NI-TX3 (+10°C) and NI-TX4 (+9°C). Furthermore, the triple mutation of 3 residues in NI-TX11 or NI-TX13 represents a much smaller modification in TrX, as compared to the substitution with the TfX sequences of 31-20 residues as in the chimeric enzymes, and this may indirectly minimize other unwanted changes in the general characteristics of the *Trichoderma* xylanase.

EXAMPLE 5

TEMPERATURE/ACTIVITY PROFILES OF MODIFIED BACILLUS XYLANASES

The assay protocol was generally identical to the one for the NI-TX xylanases in Example 4. The effect of temperature on the hydrolysis of soluble xylan in 50 mM sodium citrate buffer of pH 5.5 by the NI-BX xylanases is shown in Figure 6.

The natural BcX was active up to 60°C.

The mutant NI-BX1, with a Tfx(1-31) sequence replacing the BcX(1-22) sequence in its N-terminus, has high activity up to around 82°C. As discussed in the NI-TX section, we can define the effective temperature as a temperature allowing 150% relative activity as in 40°C. In this case, the gain in the thermophilicity of NI-BX1 would be about 22°C. The mutant NI-BX2, without the extra residues ASHA in the (-4) to the (-1) position as in NI-BX1, showed the same temperature/activity profile.

Further modification by the insertion of an Arg residues at the (-1) position in NI-BX3 and NI-BX5 has improved the thermophilicity by 2.5°C over NI-BX1 and NI-BX2.

The same gain has also been observed in NI-BX4 which has another basic residue Lys at the (-1) position. Replacement with acidic residue Glu or Asp causes a loss of around 10°C in maximum temperature (data not shown). Therefore, these results have demonstrated the contribution to the thermotolerance of the extension at the (-1) position with basic amino acids (Arg, Lys), as opposed to the presence of neutral (His) and acidic (Asp, Glu) residues.

The mutant NI-BX6 showed a gain of 2.5°C over NI-BX3 or 5°C over NI-BX1. Thus the thermophilicity can be further improved via extension of the N-terminus through the addition of the *C. acetobutylicum* xynB (23-29) sequence from the (-10) to (-4) positions, Gly at the (-3) position and Arg in both (-2) and (-1) positions.

A mutant NI-BX7 was constructed to test whether the *C. acetobutylicum* xynB (23-31) sequence in upstream extension of NI-BX6 can be replaced by a shorter tripeptide sequence GRR. Although both NI-BX6 and NI-BX7 seemed to have identical effective temperature or top temperature limit, the latter mutant of smaller size showed a wider temperature range for enzymatic activity than the former. As described above in the mutant NI-TX9, the same tripeptide was also successful in elevating the effective temperature of the *Trichoderma* xylanase, without using the CaX sequence.

In summary, the modifications which have successfully increased the thermophilicity of the fungal *Trichoderma* xylanase, are generally applicable in the bacterial *Bacillus* circulans xylanase.

EXAMPLE 6

COMPARISON OF THE THERMOPHILICITY OF THE NI-BX XYLANASES WITH THE CAMPBELL, ET AL PRIOR ART.

The best improved *Bacillus circulans* (BcX) mutant TS19a of the Campbell et al. prior art had modifications to amino acids 3,4,8, and 22 (according to the BcX numbering system). This modified xylanase was compared with the NI-BX xylanases using the same protocols described above, with the results shown in Figure 6. The temperature optima of the NI-BX xylanases are 8-14°C higher than that of TS19a. In addition, at optimum temperature the NI-BX6 and NI-BX7 xylanases of the present invention have 3-fold higher activity than that of Campbell, et al. This set of results demonstrates far superior performance of modified xylanases made by the present invention than with those of Campbell, et al.

EXAMPLE 7

COMPARISON OF THE THERMOPHILICITY OF NI-BX6 AND NI-BX7 WITH THE NATURAL CLOSTRIDIUM AND T. FUSCA XYLANASES

The N-terminal domain of the mutant xylanase NI-BX6 included an extension by a short sequence from the *Clostridium acetobutylicum* xynB (CaX). The N-terminal domain of the mutant xylanase NI-BX7 included an extension by the tripeptide GRR. NI-BX6 and NI-BX7 had a sequence of amino acids and substituted by a short sequence from a thermostable xylanase TfxA of *Thermomonospora fusca*. The thermophilicity of NI-BX6 and NI-BX7 were assessed in comparison with published data on these natural *Thermomonospora* and *Clostridium* xylanases.

The mutants NI-BX6 and NI-BX7, with high activity up to 85°C, are superior to the *Clostridium acetobutylicum* xynB (CaX) with a much lower temperature optimum of 43°C (Zappe et al. 1987, Zappe et al. 1990).

There was no data available on the temperature profile of *T. fusca* xylanase TfxA. The published data on the thermophilicity of the fermentation supernatant of *T. fusca* (Casimir-Schenkel et al. 1992) was available. This supernatant includes six xylanases, of which TfXA is one. The effect of temperature on the activity of NI-BX6 and NI-BX7 was measured according to the protocol described for the fermentation supernatant of *T. fusca* (Casimir-Schenkel et al. 1992). This included the addition of the mutant xylanase to xylan in 50 mM sodium phosphate buffer (pH 7) and incubation at 70, 80 and 90°C for exactly 10 min.

The data shows that the chimeric *Bacillus* xylanase demonstrated greater thermophilicity than the *T. fusca* xylanase supernatant, and hence is very likely greater than TfXA (Table 6).

This example demonstrates the surprising result that insertion of the short sequences in the chimeric modifications can increase the thermotolerance of the xylanase well beyond that of the thermophilic xylanases that are the source of the short sequences.

TABLE 6. Temperature profile of relative enzymatic activity at pH 7.0

Temperature °C	Relative activity (% of maximum)*		
	NI-BX6	NI-BX7	*T. fusca* super natant**
70	100	100	100
80	96	110	58
90	36	20	11

* The enzymatic activity at 70°C was calculated as 100%.

** Published data by Casimir-Schenkel et al. 1992 which is incorporated herein by reference.

In addition to higher temperature for enzymatic activity, some new mutants have also gained greater enzymatic activity, as compared to the wild-type enzyme. The relative activities of some NI-BX xylanases (NI-BX6, NI-BX7) at their respective temperature optima (75°C) were 4-fold as that of the wild type BcX (55°C) (see Figure 6).

In summary, the modifications which have successfully increased the thermophilicity of the fungal *Trichoderma* xylanase, are generally applicable in the bacterial *Bacillus circulans* xylanase.

EXAMPLE 8

PH/ACTIVITY PROFILES OF MODIFIED TRICHODERMA XYLANASES

This was a measure of the effect of different pH on the enzymatic activity of the xylanase in the hydrolysis of soluble xylan. The procedure was identical to the standard assay with changes in the incubation temperature and time. The *Trichoderma* enzymes (15 µg/mL) and soluble xylanase in 50 mM sodium citrate buffers of pH 4-8 were incubated together at 65°C. After 30 min, the amount of reducing sugars released from xylan was determined by HBAH and was expressed as a relative activity, with the value at pH optimum as 100%.

The effect of pH on the enzymatic activity of different NI-TX xylanases was shown in Figure 7.

The natural TrX, NI-TX1, NI-TX5, NI-TX6 showed the same pH/activity profile, with high activity up to pH 5.5 and a significant loss of activity at pH 6.

The substitution with Tfx(1-29) in the mutants NI-TX2, NI-TX3 and NI-TX8 results in full activity up to pH 6.0, and significant activity at pH 7. This represents a gain of 1 pH unit over the natural TrX. The mutant NI-TX9 demonstrated a pH-activity profile identical to NI-TX8, thus indicating the upstream CaX sequence can be replaced by the tripeptide GRR.

The analogue NI-TX10 with the double mutation (N10H N11D) showed a gain of 0.5 pH unit in its upper limit for activity as compared to its precursor NI-TX1. NI-TX11, NI-TX12 and NI-TX13 showed a gain of 0.6 pH unit.

EXAMPLE 9

PH PROFILES OF MODIFIED BACILLUS XYLANASES

The test procedure was identical to the protocol for the modified *Trichoderma* xylanases in Example 8. The *Bacillus circulans* xylanases were incubated with soluble xylan at 65°C in sodium citrate (pH 4-8) and sodium borate buffers (pH 9.5 and 10). As shown in Figure 8, the natural BcX is fully active up to pH 6.0.

The substitution of longer sequence with Tfx(1-33) in the mutants NI-BX1, NI-BX2, NI-BX3, NI-BX5, NI-BX6, and NI-BX7 has extended the activity to above pH 7.0 (Figure 8). This represents a gain of 1.5-2 pH units over BcX.

EXAMPLE 10

COMPARISON OF THE PH/ACTIVITY PROFILE OF THE MODIFIED BACILLUS XYLANASES WITH THAT OF CAMPBELL, ET AL

The pH range of the best xylanase TS19a of the prior art of Campbell et al. was measured and compared with the NI-BX xylanase according to the protocol described in Example 8. The results are shown in Figure 8. The pH optima of the NI-BX xylanases are 1-1.5 unit higher than that of TS19a, while TS19a itself has only a minor improvement (0.5 pH unit) over natural BcX. This result demonstrates the superior performance of the xylanases of the present invention relative to those of Campbell, et al.

EXAMPLE 11

COMPARISON OF THE PH/ACTIVITY PROFILES OF THE MODIFIED *BACILLUS* XYLANASES WITH THE NATURAL XYLANASES OF *CLOSTRIDIUM ACETOBUTYLICUM* AND *T. FUSCA*.

Since the N-terminal domain of the NI-BX xylanases was constituted by short sequences from the *Clostridium acetobutylicum* xynB (CaX) and the xylanase TfxA of *Thermomonospora fusca*, the pH ranges of the modified *Bacillus* xylanases was compared to the published data of these two natural xylanases.

The NI-BX mutant xylanases, with maximal activity up to pH 7 at 65°C (Figure 8), has a higher maximum pH than the *Clostridium acetobutylicum* xynB (CaX) with a pH optimum of 6.0 at 43°C (Zappe et al. 1987, Zappe et al. 1990).

As for the *T. fusca* xylanase TfxA, its pH range has already been disclosed (Wilson et al, PCT/ 1995). For comparative study, the effect of pH on the enzymatic activity of NI-BX xylanases was therefore determined according to the protocol as described for TfxA (Wilson et al, PCT/1995). This included the addition of the xylanase to xylan in 0.05 M sodium glycine buffers of pH 8-10 and incubation at 50°C for 30 min. As shown in Figure 9, the *Bacillus* xylanases NI-BX1, NI-BX2, NI-BX3, NI-BX6, and NI-BX7 all showed pH optima of 9 while the pH optimum of TfxA was 8.

This example shows the surprising result that the xylanases of the present invention have a higher pH range than either of the xylanases used to donate short sequences of amino acids.

EXAMPLE 12

THERMOSTABILITY OF MODIFIED *TRICHODERMA* XYLANASES

This was a measure of the tolerance of xylanase to storage at a set temperature, without any xylan present. The following parameters were generally used in both NI-TX and NI-BX xylanases. The xylanase (150 µg/mL) in assay buffer (50 mM sodium citrate, pH 5.5 or 4.5) was incubated at a set temperature. Aliquots were removed at set intervals. After cooling to 20°C, the residual enzymatic activity of the heated samples was determined via the HBAH assay of Example 3 at 40°C. The enzymatic activity was normalized as a percentage of the activity of the "0 min" aliquot.

Figure 10 shows that chimeric xylanase NI-TX5, NI-TX10, and NI-TX11 have an improved storage stability at 53 °C over the natural TrX. The replacement of the (26-29) region by the SMEL tetrapeptide sequence of Tfx in the case of NI-TX5 has allowed it to retain all of its enzymatic activity after incubation for 60 mm.

NI-TX1, TvX(3-190), and Trx expressed in *E. coli* show no improvement m thermostability over the natural TrX.

At a higher incubation temperature of 68°C, the natural TrX, NI-TX1 and NI-TX5 lost all enzymatic activity in less than 10 min (FIG. 11).

At the same temperature, the chimeric xylanase NI-TX2 has retained 40% of enzymatic activity after 10 min.

The chimeric xylanase NI-TX8 has kept 55% of its activity after 60 min, thus representing a tolerance of storage temperature which is about 15°C higher than that by the natural TrX.

In summary, the chimeric modifications in NI-TX2, NI-TX8, and NI-TX9 ie. the replacement of the (1-29) region of the *Trichoderma* xylanase by the Tfx(1-29) sequence, the upstream extension with either the *C. acetobutylicum* xynB (23-31) sequence from the (-10) to (-2) positions and Arg at the (-1) position, or the tripeptide GRR have further increased the thermostability.

EXAMPLE 13

THERMOSTABILITY OF MODIFIED *BACILLUS* XYLANASES

The NI-BX modified Bacillus xylanases and BcX were incubated at 70°C and pH 5.5 (Figure 12). The natural xylanase BcX lost all enzymatic activity as expected in less than 10 min. In contrast, the mutant xylanase NI-BX1 retained

most of the activity after 20 to 30 min. This indicated that the thermostability of the *Bacillus circulans* xylanase can be increased via the replacement of the BcX(1-22) sequence by the Tfx(1-31) sequence. Further increase in thermostability among NI-BX3, NI-BX5, NI-BX6, and NI-BX7 was achieved by the extension of the N-terminus through either the addition of the *C. acetobutylicum* xynB (23-29) sequence from the (-10) to (-4) positions, Gly at the (-3) position and Arg in both (-2) and (-1) positions or the tripeptide GRR. For NI-BX6 and NI-BX7, the gain in thermostability is about 15°C relative to the natural enzyme BcX. Thus the modifications that successfully increase the thermostability of the fungal *Trichoderma* xylanase are generally applicable in the *Bacillus* circulans xylanase .

EXAMPLE 14

EVALUATION OF PERFORMANCE OF MODIFIED TRICHODERMA XYLANASES IN TREATMENT OF PULP

The assay described above in the Examples 3 through 13 involves hydrolysis of soluble xylan and this procedure has been successful in the identification of thermophilic modified xylanases in the NI-TX and NI-BX series. However, in the pretreatment of pulp in the bleaching process, xylanase would interact with the insoluble xylan in pulp. It is therefore important to confirm that the performance improvements identified using the soluble xylan substrate would be observed in the treatment of pulp. Therefore, the performance of the modified xylanases was evaluated in the treatment of brown-stock pulp prior to bleaching.

Samples of natural and modified enzymes were sent to Iogen Corporation of Ottawa, Canada for testing on pulp. Iogen manufactures and supplies xylanase enzymes to the pulp industry and has developed tests to assess the performance of xylanase enzymes in treating pulp. The test involves adding the enzymes to pulp for a specified period and then measuring the effect of the enzyme on the subsequent bleachability of the pulp. The test were carried out using a commercial softwood Kraft pulp of Kappa number 25.6 .

The results of the Iogen pulp testing of *Trichoderma* xylanases is shown in Figure 13. At pH 6.0, the optimum pH for the natural TrX in treating pulp, good performance was achieved up to 52°C. The natural TrX was not active on pulp at pH 6.5 .

The *E. coli*-expressed NI-TX1 with a single point mutation Q162H was ineffective on pulp at temperatures higher than 40°C. By contrast, the mutants NI-TX5, NI-TX2 and NI-TX8 could function up to 60, 61, and 65°C respectively at pH 6.5.

Although the absolute temperatures tolerated by the enzymes are lower on pulp than in the hydrolysis of soluble xylan, the improvements by the modified xylanases in temperature (+8 to13°C) and pH (+0.5 unit) in the treatment of pulp were consistent with the gains in their thermophilicity and alkalophilicity in xylan hydrolysis described in Examples 4 and 8.

The temperature and pH ranges tolerated by the enzymes in pulp treatment is encouraging, based on these preliminary tests. Much more extensive testing of temperature and pH range using different pulps and treatment techniques would likely increase the range of conditions in which the modified xylanases are effective on pulp.

EXAMPLE 15

EVALUATION OF PERFORMANCE OF MODIFIED BACILLUS XYLANASES IN TREATMENT OF PULP

The *B. circulans* xylanases were also tested in the treatment of pulp by Iogen Corporation (Figure 14).

At pH 8.0, the natural BcX showed poor performance at temperatures higher than 40°C. When the pH was lowered to 7, BcX was only effective up to 50°C.

By contrast, the mutant NI-BX1 was active up to 75°C at pH 8.0.

Although the absolute temperature tolerated by the NI-BX1 enzyme is lower on pulp than in the hydrolysis of soluble xylan, the improvements by the modified xylanase in temperature (+28°C) and pH (+1 unit) in the treatment of pulp were consistent with the gains in their temperature tolerance and pH range in xylan hydrolysis described in Examples 5 and 9.

The temperature and pH ranges tolerated by the enzymes in pulp treatment is encouraging, based on these preliminary tests. Much more extensive testing of temperature and pH range using different pulps and treatment techniques would likely increase the range of conditions in which the modified xylanases are effective on pulp.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: NATIONAL RESEARCH COUNCIL OF CANADA (Canada Crown corporation)
(B) STREET: 1200 Montreal Road
(C) CITY: Ottawa
(D) STATE: Ontario
(E) COUNTRY: CANADA
(F) POSTAL CODE (ZIP): K1A 0R6

(ii) TITLE OF INVENTION: MODIFICATION OF XYLANASE TO IMPROVE THERMOPHILICITY, ALKOPHILICITY AND THERMOSTABILITY

(iii) NUMBER OF SEQUENCES: 54

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(B) COMPUTER: IBM PC compatible
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 184 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus niger, var. awamori

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Moat Dr, J
Dr Roga, M
Dr Verbakel, J
Stam, H
Santos da Silva, M J
Egmond, M R
Hagemans, M.L. D
Gorcom, R.F.M.V.
Hessing, J.G.M.
Hondel, C.A.M.J
(C) JOURNAL: Xylan and Xylanase
(F) PAGES: 349-360
(G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Asp Thr Arg Ile Asn Glu Pro Ser Ile Thr Gly Thr Ser Thr Phe Thr
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Gln Tyr Phe Ser Val Arg Glu Ser Thr Arg Thr Ser Gly Thr Val Thr
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 185 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus tubigensis

(x) PUBLICATION INFORMATION:
(A) AUTHORS: de Graaff, L.H.
van der Broeck, H.C.

van Ooijan, A.J.J.
Visser, J
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 185 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus circulans

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Yang, R.C.A.
MacKenzie, C.R.
Narang, S.A.
(C) JOURNAL: Nucleic Acid Research
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
20 25 30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
35 40 45

Arg Thr Ile Asn Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Gly
50 55 60

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
65 70 75 80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
85 90 95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
100 105 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
115 120 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
130 135 140

Thr Phe Thr Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145 150 155 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
165 170 175

Ser Gly Ser Ser Asn Val Thr Val Trp
180 185

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 201 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus pumilus

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Fukusaki, E
Panbangred, W
Shinmyo, A
Okada, H
(C) JOURNAL: FEBS Letters
(D) VOLUME: 171
(F) PAGES: 197-201
(G) DATE: 1984

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Arg Thr Ile Thr Asn Asn Glu Met Gly Asn His Ser Gly Tyr Asp Tyr
1               5                  10                  15

Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ser Met Thr Leu Asn Asn Gly
            20                  25                  30

Gly Ala Phe Ser Ala Gly Trp Asn Asn Ile Gly Asn Ala Leu Phe Arg
        35                  40                  45

Lys Gly Lys Lys Phe Asp Ser Thr Arg Thr His His Gln Leu Gly Asn
    50                  55                  60

Ile Ser Ile Asn Tyr Asn Ala Ser Phe Asn Pro Ser Gly Asn Ser Tyr
65                  70                  75                  80

Leu Cys Val Tyr Gly Trp Thr Gln Ser Pro Leu Ala Glu Tyr Tyr Ile
                85                  90                  95

Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Ala Tyr Lys Gly Ser
            100                 105                 110

Phe Tyr Ala Asp Gly Gly Thr Tyr Asp Ile Tyr Glu Thr Thr Arg Val
        115                 120                 125

Asn Gln Pro Ser Ile Ile Gly Ile Ala Thr Phe Lys Gln Tyr Trp Ser
    130                 135                 140

Val Arg Gln Thr Lys Arg Thr Ser Gly Thr Val Ser Val Ser Ala His
145                 150                 155                 160

Phe Arg Lys Trp Glu Ser Leu Gly Met Pro Met Gly Lys Met Tyr Glu
                165                 170                 175

Thr Ala Phe Thr Val Glu Gly Tyr Gln Ser Ser Gly Ser Ala Asn Val
            180                 185                 190

Met Thr Asn Gln Leu Phe Ile Gly Asn
        195                 200
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 185 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus subtilus

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Parce, M.G.
Bourbonnais, R
Desrochers, M
Jurasek, L
Yaguchi, M
(C) JOURNAL: Arch. Microbiol.
(D) VOLUME: 144
(F) PAGES: 201-206
(G) DATE: 1986

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Ile Val
1               5                  10                  15

Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
            20                  25                  30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
        35                  40                  45

Arg Thr Ile Asn Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Gly
    50                  55                  60

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
65                  70                  75                  80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
                85                  90                  95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
            100                 105                 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
        115                 120                 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
    130                 135                 140

Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145                 150                 155                 160
```

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
165 170 175

Ser Gly Ser Ser Asn Val Thr Val Trp
180 185

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 211 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Clostridium acetobutylicum P262
(B) STRAIN: Xyn B

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Zappe, H
Jones, W. A.
Woods, D. R.
(C) JOURNAL: Nucleic Acids Res.
(D) VOLUME: 18
(F) PAGES: 2719
(G) DATE: 1990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Ala Phe Asn Thr Gln Ala Ala Pro Lys Thr Ile Thr Ser Asn Glu
1 5 10 15

Ile Gly Val Asn Gly Gly Tyr Asp Tyr Glu Leu Trp Lys Asp Tyr Gly
20 25 30

Asn Thr Ser Met Thr Leu Lys Asn Gly Gly Ala Phe Ser Cys Gln Trp
35 40 45

Ser Asn Ile Gly Asn Ala Leu Phe Arg Lys Gly Lys Lys Phe Asn Asp
50 55 60

Thr Gln Thr Tyr Lys Gln Leu Gly Asn Ile Ser Val Asn Tyr Asn Cys
65 70 75 80

Asn Tyr Gln Pro Tyr Gly Asn Ser Tyr Leu Cys Val Tyr Gly Trp Thr
85 90 95

Ser Ser Pro Leu Val Glu Tyr Tyr Ile Val Asp Ser Trp Gly Ser Trp
100 105 110

Arg Pro Pro Gly Gly Thr Ser Lys Gly Thr Ile Thr Val Asp Gly Gly
115 120 125

Ile Tyr Asp Ile Tyr Glu Thr Thr Arg Ile Asn Gln Pro Ser Ile Gln
130 135 140

Gly Asn Thr Thr Phe Lys Gln Tyr Trp Ser Val Arg Arg Thr Lys Arg
145 150 155 160

Thr Ser Gly Thr Ile Ser Val Ser Lys His Phe Ala Ala Trp Glu Ser
165 170 175

Lys Gly Met Pro Leu Gly Lys Met His Glu Thr Ala Phe Asn Ile Glu
180 185 190

Gly Tyr Gln Ser Ser Gly Lys Ala Asp Val Asn Ser Met Ser Ile Asn
195 200 205

Ile Gly Lys
210

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 206 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Clostridium stercorarium
(B) STRAIN: Xyn A

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Sakka, K
Kojima, Y
Kondo, T
Karita, S
Ohmiya, K
Shimada, K
(C) JOURNAL: Biosci, Biotech, Biochem
(D) VOLUME: 57
(F) PAGES: 273-277
(G) DATE: 1993

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Arg Ile Ile Tyr Asp Asn Glu Thr Gly Thr His Gly Gly Tyr Asp
1 5 10 15

Tyr Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ile Met Glu Leu Asn Asp
20 25 30

```
Gly Gly Thr Phe Ser Cys Gln Trp Ser Asn Ile Gly Asn Ala Leu Phe
        35                  40                  45

Arg Lys Gly Arg Lys Phe Asn Ser Asp Lys Thr Tyr Gln Glu Leu Gly
    50                  55                  60

Asp Ile Val Val Glu Tyr Gly Cys Asp Tyr Asn Pro Asn Gly Asn Ser
65                  70                  75                  80

Tyr Leu Cys Val Tyr Gly Trp Thr Arg Asn Phe Leu Val Glu Tyr Tyr
                85                  90                  95

Ile Val Glu Ser Trp Gly Ser Trp Arg Pro Pro Gly Ala Thr Pro Lys
            100                 105                 110

Gly Thr Ile Thr Gln Trp Met Ala Gly Thr Tyr Glu Ile Tyr Glu Thr
        115                 120                 125

Thr Arg Val Asn Gln Pro Ser Ile Asp Gly Thr Ala Thr Phe Gln Gln
    130                 135                 140

Tyr Trp Ser Val Arg Thr Ser Lys Arg Thr Ser Gly Thr Ile Ser Val
145                 150                 155                 160

Thr Glu His Phe Lys Gln Trp Glu Arg Met Gly Met Arg Met Gly Lys
                165                 170                 175

Met Tyr Glu Val Ala Leu Thr Val Glu Gly Tyr Gln Ser Ser Gly Tyr
            180                 185                 190

Ala Asn Val Tyr Lys Asn Glu Ile Arg Ile Gly Ala Asn Pro
        195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 211 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: Ruminococcus flavefaciens

(x) PUBLICATION INFORMATION:
- (A) AUTHORS: Zhang, J
  Flint, H. J.
- (C) JOURNAL: EMBL database accession number Z11127
- (G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Ser Ala Ala Asp Gln Gln Thr Arg Gly Asn Val Gly Gly Tyr Asp Tyr
1               5                  10                  15

Glu Met Trp Asn Gln Asn Gly Gln Gly Gln Ala Ser Met Asn Pro Gly
            20                  25                  30

Ala Gly Ser Phe Thr Cys Ser Trp Ser Asn Ile Glu Asn Phe Leu Ala
        35                  40                  45

Arg Met Gly Lys Asn Tyr Asp Ser Gln Lys Lys Asn Tyr Lys Ala Phe
    50                  55                  60

Gly Asn Ile Val Leu Thr Tyr Asp Val Glu Tyr Thr Pro Arg Gly Asn
65                  70                  75                  80

Ser Tyr Met Cys Val Tyr Gly Trp Thr Arg Asn Pro Leu Met Glu Tyr
                85                  90                  95

Tyr Ile Val Glu Gly Trp Gly Asp Trp Arg Pro Pro Gly Asn Asp Gly
            100                 105                 110

Glu Val Lys Gly Thr Val Ser Ala Asn Gly Asn Thr Tyr Asp Ile Arg
        115                 120                 125

Lys Thr Met Arg Tyr Asn Gln Pro Ser Leu Asp Gly Thr Ala Thr Phe
    130                 135                 140

Pro Gln Tyr Trp Ser Val Arg Gln Thr Ser Gly Ser Ala Asn Asn Gln
145                 150                 155                 160

Thr Asn Tyr Met Lys Gly Thr Ile Asp Val Ser Lys His Phe Asp Ala
                165                 170                 175

Trp Ser Ala Ala Gly Leu Asp Met Ser Gly Thr Leu Tyr Glu Val Ser
            180                 185                 190

Leu Asn Ile Glu Gly Tyr Arg Ser Asn Gly Ser Ala Asn Val Lys Ser
        195                 200                 205

Val Ser Val
    210
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 197 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Schizophyllum commune
(B) STRAIN: Xylanase A

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Oku, T
Yaguchi, M
Parse, M
Jurasek, L
(C) JOURNAL: Canadian Fed. Biol. Soc. annual meeting
(F) PAGES: Abstract #676
(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser Gly Thr Pro Ser Ser Thr Gly Thr Asp Gly Gly Tyr Tyr Tyr Ser
1 5 10 15

Trp Trp Thr Asp Gly Ala Gly Asp Ala Thr Tyr Gln Asn Asn Gly Gly
20 25 30

Gly Ser Tyr Thr Leu Thr Trp Ser Gly Asn Asn Gly Asn Leu Val Gly
35 40 45

Gly Lys Gly Trp Asn Pro Gly Ala Ala Ser Arg Ser Ile Ser Tyr Ser
50 55 60

Gly Thr Tyr Gln Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp
65 70 75 80

Thr Arg Ser Ser Leu Ile Glu Tyr Tyr Ile Val Glu Ser Tyr Gly Ser
85 90 95

Tyr Asp Pro Ser Ser Ala Ala Ser His Lys Gly Ser Val Thr Cys Asn
100 105 110

Gly Ala Thr Tyr Asp Ile Leu Ser Thr Trp Arg Tyr Asn Ala Pro Ser
115 120 125

Ile Asp Gly Thr Gln Thr Phe Glu Gln Phe Trp Ser Val Arg Asn Pro
130 135 140

Lys Lys Ala Pro Gly Gly Ser Ile Ser Gly Thr Val Asp Val Gln Cys
145 150 155 160

His Phe Asp Ala Trp Lys Gly Leu Gly Met Asn Leu Gly Ser Glu His
165 170 175

Asn Tyr Gln Ile Val Ala Thr Glu Gly Tyr Gln Ser Ser Gly Thr Ala
180 185 190

Thr Ile Thr Val Thr
195

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 191 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Streptomyces lividans
(B) STRAIN: Xln B

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Shareck, F
Roy, C
Yaguchi, M
Morosoli, R
Kluepfel, D
(C) JOURNAL: Gene
(D) VOLUME: 107
(F) PAGES: 75-82
(G) DATE: 1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Asp Thr Val Val Thr Thr Asn Gln Glu Gly Thr Asn Asn Gly Tyr Tyr
1               5                  10                  15

Tyr Ser Phe Trp Thr Asp Ser Gln Gly Thr Val Ser Met Asn Met Gly
            20                  25                  30

Ser Gly Gly Gln Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val
        35                  40                  45

Ala Gly Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Gln Tyr Ser
    50                  55                  60

Gly Ser Phe Asn Pro Ser Gly Asn Ala Tyr Leu Ala Leu Tyr Gly Trp
65                  70                  75                  80

Thr Ser Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Thr
                85                  90                  95

Tyr Arg Pro Thr Gly Glu Tyr Lys Gly Thr Val Thr Ser Asp Gly Gly
            100                 105                 110

Thr Tyr Asp Ile Tyr Lys Thr Thr Arg Val Asn Lys Pro Ser Val Glu
        115                 120                 125

Gly Thr Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg
    130                 135                 140

Thr Gly Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145                 150                 155                 160

Ala Gly Met Pro Leu Gly Asn Phe Ser Tyr Tyr Met Ile Asn Ala Thr
                165                 170                 175
```

Glu Gly Tyr Gln Ser Ser Gly Thr Ser Ser Ile Asn Val Gly Gly

180 185 190

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 191 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Streptomyces lividans
(B) STRAIN: Xln C

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Shareck, F
Roy, C
Yaguchi, M
Morosoli, R
Kluepfel, D
(C) JOURNAL: Gene
(D) VOLUME: 107
(F) PAGES: 75-82
(G) DATE: 1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Thr Thr Ile Thr Thr Asn Gln Thr Gly Thr Asp Gly Met Tyr Tyr
1 5 10 15

Ser Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly
20 25 30

Gly Gly Ser Tyr Ser Thr Gln Trp Thr Asn Cys Gly Asn Phe Val Ala
35 40 45

Gly Lys Gly Trp Ser Thr Gly Asp Gly Asn Val Arg Tyr Asn Gly Tyr
50 55 60

Phe Asn Pro Val Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
85 90 95

Pro Thr Gly Thr Tyr Lys Gly Thr Val Ser Ser Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Gln Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Gly Thr
115 120 125

Lys Thr Phe Gln Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
130 135 140

Gly Ser Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145 150 155 160

Ala Gly Met Asn Met Gly Gln Phe Arg Tyr Tyr Met Ile Asn Ala Thr
165 170 175

Glu Gly Tyr Gln Ser Ser Gly Ser Ser Asn Ile Thr Val Ser Gly
180 185 190

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 189 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Streptomyces sp. 36a

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Nagashima, M
Okumoto, Y
Okanishi, M
(C) JOURNAL: Trends in Actinomycetologia
(F) PAGES: 91-96
(G) DATE: 1989

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Thr Thr Ile Thr Asn Glu Thr Gly Tyr Asp Gly Met Tyr Tyr Ser
1 5 10 15

Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly Gly
20 25 30

Gly Ser Tyr Ser Thr Arg Trp Thr Asn Cys Gly Asn Phe Val Ala Gly
35 40 45

Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Arg Tyr Thr Gly Trp
50 55 60

Phe Asn Pro Ser Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
85 90 95

```
Pro Thr Gly Glu Thr Arg Gly Thr Val His Ser Asp Gly Gly Thr Tyr
                100                 105                 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Ala Pro
        115                 120                 125

Ala Ala Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
    130                 135                 140

Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg Ala Gly
145                 150                 155                 160

Met Asn Met Gly Asn Phe Arg Tyr Tyr Met Ile Asn Ala Thr Glu Gly
                165                 170                 175

Tyr Gln Ser Ser Gly Ser Ser Thr Ile Thr Val Ser Gly
            180                 185
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 189 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: Thermomonospora fusca
- (B) STRAIN: Tfx A

(x) PUBLICATION INFORMATION:
- (A) AUTHORS: Irwin, D
  Jung, E. D.
  Wilson, D. B.
- (C) JOURNAL: Appl. Environ. Microbiol.
- (D) VOLUME: 60
- (F) PAGES: 763-770
- (G) DATE: 1994

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Ala Val Thr Ser Asn Glu Thr Gly Tyr His Asp Gly Tyr Phe Tyr Ser
1               5                   10                  15

Phe Trp Thr Asp Ala Pro Gly Thr Val Ser Met Glu Leu Gly Pro Gly
            20                  25                  30

Gly Asn Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val Ala Gly
        35                  40                  45

Lys Gly Trp Ala Thr Gly Gly Arg Arg Thr Val Thr Tyr Ser Ala Ser
    50                  55                  60
```

```
Phe Asn Pro Ser Gly Asn Ala Tyr Leu Thr Leu Tyr Gly Trp Thr Arg
65                  70                  75                  80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Glu Ser Trp Gly Thr Tyr Arg
                85                  90                  95

Pro Thr Gly Thr Tyr Met Gly Thr Val Thr Thr Asp Gly Gly Thr Tyr
            100                 105                 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Ile Glu Gly Thr
        115                 120                 125

Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg Thr Ser
    130                 135                 140

Gly Thr Ile Thr Ala Gly Asn His Phe Asp Ala Trp Ala Arg His Gly
145                 150                 155                 160

Met His Leu Gly Thr His Asp Tyr Met Ile Met Ala Thr Glu Gly Tyr
                165                 170                 175

Gln Ser Ser Gly Ser Ser Asn Val Thr Leu Gly Thr Ser
            180                 185
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 190 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: Thrichoderma harzianum

(x) PUBLICATION INFORMATION:
- (A) AUTHORS: Yaguchi, M
  Roy, C
  Watson, D. C.
  Rollin, F
  Tan, L. U. L.
  Senior, D. J.
  Saddler, J. N.
- (C) JOURNAL: Xylan and Xylanase
- (F) PAGES: 435-438
- (G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Gln Thr Ile Gly Pro Gly Thr Gly Tyr Ser Asn Gly Tyr Tyr Tyr Ser
1               5                   10                  15
```

```
Tyr Trp Asn Asp Gly His Ala Gly Val Thr Tyr Thr Asn Gly Gly Gly
            20                  25                  30

Gly Ser Phe Thr Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
        35                  40                  45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
    50                  55                  60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Ile Tyr Gly Trp Ser
65                  70                  75                  80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
                85                  90                  95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
            100                 105                 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
        115                 120                 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
    130                 135                 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145                 150                 155                 160

Ser His Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
                165                 170                 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
            180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 178 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: Trichoderma reesei
- (B) STRAIN: Xyn I

(x) PUBLICATION INFORMATION:
- (A) AUTHORS: Torronene, A
  Mach, R. L.
  Messner, R.
  Gonzalez, R.
  Kalkkinen, N

Harkki, A
Kubicek, C. P.
(C) JOURNAL: BioTechnology
(D) VOLUME: 10
(F) PAGES: 1461-1465
(G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Ala Ser Ile Asn Tyr Asp Gln Asn Tyr Gln Thr Gly Gly Gln Val Ser
1               5                  10                  15

Tyr Ser Pro Ser Asn Thr Gly Phe Ser Val Asn Trp Asn Thr Gln Asp
            20                  25                  30

Asp Phe Val Val Gly Val Gly Trp Thr Thr Gly Ser Ser Ala Pro Ile
        35                  40                  45

Asn Phe Gly Gly Ser Phe Ser Val Asn Ser Gly Thr Gly Leu Leu Ser
    50                  55                  60

Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
65                  70                  75                  80

Asp Asn His Asn Tyr Pro Ala Gln Gly Thr Val Lys Gly Thr Val Thr
                85                  90                  95

Ser Asp Gly Ala Thr Tyr Thr Ile Trp Glu Asn Thr Arg Val Asn Glu
            100                 105                 110

Pro Ser Ile Gln Gly Thr Ala Thr Phe Asn Gln Tyr Ile Ser Val Arg
        115                 120                 125

Asn Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His Phe Asn
    130                 135                 140

Trp Ala Ser Leu Gly Leu His Leu Gly Gln Met Met Asn Tyr Gln Val
145                 150                 155                 160

Val Ala Val Glu Gly Trp Gly Gly Ser Gly Ser Ala Ser Gln Ser Val
                165                 170                 175

Ser Asn
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 190 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Trichoderma reesei
(B) STRAIN: Xyn II

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Torronene, A
Mach, R. L.
Messner, R
Gonzalez, R
Kalkkinen, N
Harkki, A
Kubicek, C. P.
(C) JOURNAL: Biotechnology
(D) VOLUME: 10
(F) PAGES: 1461-1465
(G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gln Thr Ile Gln Pro Gly Thr Gly Tyr Asn Asn Gly Tyr Phe Tyr Ser
1 5 10 15

Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
20 25 30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
180 185 190

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 190 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Trichoderma viride

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Yaguchi, M
Roy, C
Ujie, M
Watson, D. C.
Wakarchuk, W.
(C) JOURNAL: Xylan and Xylanase
(F) PAGES: 149-154
(G) DATE: 1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gln Thr Ile Gln Pro Gly Thr Gly Phe Asn Asn Gly Tyr Phe Tyr Ser
1 5 10 15

Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
20 25 30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Thr His
130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser

180 185 190

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 573 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: pTvX(3-190)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTAGCATAGG ACCAGGAACC GGTTTCAACA ACGGTTACTT TTACAGCTAT TGGAACGATG 60

GCCATGGTGG TGTTACCTAT ACAAACGGGC CCGGAGGCCA ATTTAGCGTC AATTGGTCTA 120

ACTCCGGAAA CTTCGTAGGT GGAAAAGGTT GGCAACCCGG GACCAAAAAT AAGGTGATCA 180

ACTTCTCTGG ATCTTATAAT CCGAATGGGA ATTCATACTT AAGCGTCTAT GGCTGGTCTA 240

GAAACCCACT GATTGAATAT TACATTGTCG AAAATTTCGG TACCTACAAT CCGAGTACCG 300

GCGCCACAAA ATTAGGCGAA GTCACTAGTG ATGGATCCGT ATATGATATC TACCGTACCC 360

AACGCGTTAA TCAGCCATCG ATCATTGGAA CCGCCACCTT TTATCAGTAC TGGAGTGTTA 420

GACGTACGCA TCGGAGCTCC GGTTCGGTTA ATACTGCGAA TCACTTTAAT GCATGGGCAC 480

AGCAAGGGTT AACCCTAGGT ACAATGGATT ATCAAATCGT AGCGGTGGAA GGCTACTTCT 540

CGAGTGGTTC CGCTAGTATT ACAGTGAGCT AAA 573

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 579 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: pXYbc

(viii) POSITION IN GENOME:
(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCTAGCACAG ATTACTGGCA AAACTGGACA GACGGTGGCG GTATCGTTAA TGCCGTGAAC 60

GGCTCCGGAG GCAACTACAG CGTGAATTGG TCTAATACTG GGAACTTCGT AGTCGGAAAA 120

GGTTGGACGA CAGGATCCCC GTTCCGTACG ATCAACTACA ACGCTGGCGT TTGGGCCCCG 180

AATGGTAACG GTTACCTGAC ACTGTATGGC TGGACGCGTT CGCCACTGAT TGAATATTAC 240

GTTGTCGACT CTTGGGGAAC GTACCGTCCG ACTGGAACCT ACAAAGGCAC AGTCAAAAGC 300

GATGGTGGTA CCTATGACAT CTACACCACC ACAAGATACA ACGCACCTTC CATCGATGGC 360

GATCGGACCA CCTTTACTCA GTATTGGAGT GTTAGACAAT CTAAGCGGCC GACTGGTTCG 420

AACGCCACCA TTACGTTCAC CAATCACGTG AATGCATGGA AATCCCACGG TATGAACCTA 480

GGTTCTAATT GGGCTTATCA AGTAATGGCG ACCGAAGGCT ACCAGAGCTC TGGTTCTTCC 540

AACGTTACAG TGTGGTAAAG ATCTTGAAGC TTGGGACGT 579

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-162H-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGGGCACAGC ACGGGTTAAC C 21

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-162H-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTAGGGTTAA CCCGTGCTGT GCCCATGCA 29

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTAGCCACGC GGCCGTAACT TCAAATGAAA CCGGTTATCA TGACGGCTAT TTCTACAGCT 60

TCTGG 65

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ACCGATGCAC CGGGAACTGT GTCCATGGAG CTCGGGCC 38

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCATGATAAC CGGTTTCATT TGAAGTTACG GCCGCGTGG 39

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CGAGCTCCAT GGACACAGTT CCCGGTGCAT CGGTCCAGAA GCTGTAGAAA TAGCCG 56

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx(1-6)-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CTAGCTAAGG AGGCTGCAGA TGGCAGTAAC ATCAAATGAA A 41

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx(1-6)-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCGGTTTCAT TTGATGTTAC TGCCATCTGC AGCCTCCTTA G 41

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-1f

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTAGCTAAGG AGGCTGCAGA TGCAAACAAT ACAACCAGGA A 41

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-8f

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCGGTTCCTG GTTGTATTGT TTGCATCTGC AGCCTCCTTA G 41

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-26SMEL-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CATGGTGGTG TGAGCATGGA GCTCGGGCC 29

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-28E/29L-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CGAGCTCGTA GGTCACACCA C 21

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-27M/29L-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CATGGAGGCG TCACAATGAC TCTGGGGCC 29

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-27M/29L-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCAGAGTCAT TGTGACGCCT C 21

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-27M-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CATGGAGGCG TCACAATGAC TAATGGGCC 29

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX-27M-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CATTAGTCAT TGTGACGCCT C 21

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)R-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CTAGCGCAAG AGCAGTAACA AGTAACGAGA 30

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)R-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCGGTCTCGT TACTTGTTAC TGCTCTTGCG 30

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: CaTf-(-1)R-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CTAGCGCATT CAACACACAG GCCGCTCCTC GAGCTGTCAC CAGCAAC 47

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: CaTf-(-1)R2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCGGTCTCGT TGCTGGTGAC AGCTCGAGGA GCGGCCTGTG TGTTGAATGC G 51

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: GRR-Tf(1-6)-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

ACTCTGCAGA TGGGAAGAAG GGCCGTAACT TCAAATGAAA CCGGTTATCA TGAC 54

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Uni-PCR-1r

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GAAAAGTGCC ACCTGACGTC CCAAGCTT 28

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX10HD-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CCGGTTTCCA CGACGGTTAC TTTTACAGCT ATTGGAACGA CGGCCATGGA GGAGTAACTT 60

ACACCAATGG GCC 73

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX10HD-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CATTGGTGTA AGTTACTCCT CCATGGCCGT CGTTCCAATA GCTGTAAAAG TAACCGTCGT 60

GGAAA 65

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TX10HD/N-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GAAACCGGTT ACCACRACGG TTACTTTTAC AGCTATTGGA ACGATGGCC 49

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TrX1f

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CTAGCTAAGG AGGCTGCAGA TGCAAACAAT ACAACCAGGA A 41

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: TrX8f

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CCGGTTCCTG GTTGTATTGT TTGCATCTGC AGCCTCCTTA G 41

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-2b

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

ACCGATGCCC CGGGAACTGT GAGTATGGAG CTCGGCC 37

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tfx-4b

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CCGGGGCCGA GCTCCATACT CACAGTTCCC GGGGCATCGG TCCAGAAGCT GTAGAAATAG 60

CCG 63

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)K-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CTAGCGCAAA AGCAGTAACA AGTAACGAGA 30

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)K-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CCGGTCTCGT TACTTGTTAC TGCTTTTGCG 30

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)D-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CTAGCGCAGA TGCAGTAACA AGTAACGAGA 30

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Tf-(-1)E-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

CCGGTCTCGT TACTTGTTAC TGCTTCTGCG 30

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: CaTf-PCR-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CCCGCTAGCG CATTCAACAC ACAAGCARGT SSAAGGGCCG TAACTTCAAA TGAAACCGGT 60

T 61

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Xy-14a

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

ATTCGGGGCC CAAACGCCAG CGTTGTAGTT GATCGTACG 39

Claims

1. A Family 11 xylanase enzyme selected from the group consisting of the Family 11 xylanases of *Trichoderma*, *Aspergillus*, *Streptomyces*, and *Bacillus* having the amino acid tyrosine or phenylalanine in position 14 as determined by the amino acid numbering of *Trichoderma reesei* xylanase II or the corresponding position in the other xylanases when the amino acids are aligned and having at least 8 amino acid residues in a N-terminus upstream from position 10, said xylanase enzyme having been modified to exhibit enhanced thermophilicity, alkalophilicity, or thermostability with respect to a naturally-occurring enzyme by replacing an amino acid at position 10 with a different amino acid.

2. A modified xylanase according to claim 1 wherein the Family 11 xylanase is selected from the group consisting of *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma harzianum* Xylanase, *Trichoderma*

viride Xylanase, *Streptomyces lividans* xylanase B, and *Streptomyces lividans* xylanase C.

3. A modified xylanase according to claim 1 wherein amino acid 10 is substituted with another acid, and amino acids 27 and 29 are substituted with amino acids selected from the group consisting of valine, methionine, isoleucine, and leucine.

4. A modified xylanase according to claim 3 wherein amino acids 10, 27, and 29 are substituted with histidine, methionine, and leucine, respectively.

5. A Family 11 xylanase enzyme selected from the group consisting of the Family 11 xylanases of *Trichoderma*, *Aspergillus*, *Streptomyces*, and *Bacillus* having the amino acid tyrosine or phenylalanine in position 14 as determined by the amino acid numbering of *Trichoderma reesei* xylanase II or the corresponding position in the other xylanases when the amino acids are aligned, said xylanase enzyme having been modified to exhibit enhanced thermophilicity, alkalophilicity, or thermostability by substituting a sequence of amino acids in a N-terminal region of said xylanase enzyme with that of a corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A to form a chimeric xylanase, and an upstream extension of the N-terminus of the chimeric xylanase with the addition of from 0 to 10 amino acids.

6. A modified xylanase according to claim 5 wherein the Family 11 xylanase is selected from the group consisting of *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma harzianum* xylanase, *Trichoderma viride* xylanase, *Bacillus circulans* xylanase A, *Bacillus subtilis* xylanase A, *Aspergillus niger* xylanase A, *Aspergillus kawachii* xylanase C, *Aspergillus tubigensis* xylanase A, *Streptomyces lividans* xylanase B, and *Streptomyces lividans* xylanase C.

7. A modified xylanase according to claim 1 wherein the sequence of amino acids comprising amino acids 10-29 is substituted by the corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A.

8. A modified xylanase according to claim 5 wherein the sequence of amino acids comprising amino acids 1-29 is substituted by the corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A.

9. A modified xylanase according to claim 5 wherein said xylanase is selected from the group consisting of NI-TX2, NI-TX3, NI-TX4, NI-TX5, NI-TX7, NI-TX8, NI-TX9, NI-BX1, NI-BX2, NI-BX3, NI-BX4, NI-BX5, NI-BX6 and NI-BX7.

10. A modified xylanase according to claim 8 wherein the upstream extension of the N-terminus consists of the addition of one or two basic amino acids in the -2 and -1 positions and the (23-31) region of *Clostridium acetobutylicum* xylanase xynB in the -10 to -3 positions.

11. A modified xylanase according to claim 5 wherein the upstream extension of the N-terminus comprises the addition of any of the tetrapeptides identified by the sequences Alanine-Serine-Alanine-Arginine or Alanine-Serine-Alanine-Lysine.

12. A modified xylanase according to claim 8 wherein the upstream extension of the N-terminus comprises the addition of the residues glycine-arginine-arginine in the (-3) to (-1) positions.

13. A method of improving the bleachability of wood pulp, the method comprising treating the pulp with a xylanase according to claim 1 for approximately 5 minutes to three hours, at a temperature between approximately 55°C to 75°C.

14. The method of claim 13 wherein the xylanase treatment is carried out at pH 7.5 to 9.0.

15. A modified xylanase according to claim 5 wherein the upstream extension of the N-terminus comprises the addition of the residues glycine-arginine-arginine in the (-3) to (-1) positions.

16. A modified xylanase according to claim 15 wherein the sequence of amino acids comprising amino acids 1-29 of *Trichoderma* xylanase II or the corresponding aligned sequence of another Family 11 xylanase is substituted by the corresponding aligned sequence of amino acids from xylanase from another Family 11 xylanase.

17. A modified xylanase according to claim 16 wherein the sequence of amino acids comprising amino acids 1-29 of

Trichoderma reesei xylanase II or the corresponding aligned sequence of another Family 11 xylanase are replaced with the corresponding aligned sequence of amino acids from *Thermomonospora fusca* xylanase A.

18. A method of modifying a Family 11 xylanase enzyme to improve any or all of the thermophilicity, alkalophilicity, and thermotolerance, the method consisting of one or more of the following three types of modifications:

(1) Modification of amino acids 10, 27, or 29 of *Trichoderma reesei* xylanase II or the corresponding aligned amino acids of another Family 11 xylanase;
(2) Substitution of one or more sequences of amino acids in the N-terminal region with that of corresponding aligned sequences of amino acids from another Family 11 xylanase to form a chimeric xylanase; and
(3) Upstream extension of the N-terminus with the addition of up to 10 amino acids.

19. A method of improving the bleachability of wood pulp, the method comprising treating the pulp with a modified Family 11 xylanase according to claim 5 for 5 minutes to three hours at 55°C to 75°C.

20. The method of claim 19 wherein the xylanase treatment is carried out at pH 7.5 to 9.0.

21. A method of improving the bleachability of wood pulp, the method comprising treating the pulp with a modified Family 11 xylanase according to claim 2 for approximately 5 minutes to three hours, at a temperature between approximately 55°C to 75°C.

22. The method of claim 21 wherein the xylanase treatment is carried out at pH 7.5 to 9.0.

23. A method of improving the bleachability of wood pulp, the method comprising treating the pulp with a modified Family 11 xylanase according to claim 6 for approximately 5 minutes to three hours, at a temperature between approximately 55°C to 75°C.

24. The method of claim 23 wherein the xylanase treatment is carried out at pH 7.5 to 9.0.

25. A modified xylanase according to claim 1 wherein said xylanase is selected from the group consisting of NI-TX6, NI-TX10, NI-TX11, NI-TX12 or NI-TX13.

Figure 1

Abbreviations of species

Bp	*Bacillus pumilus*
Ca	*Clostridium acetobutylicum* P262 XynB
Cs	*Clostridium stercorarium* xynA
Rf	*Ruminococcus flavefaciens*
Tr2	*Trichoderma reesei* XYN II
Tv	*Trichoderma viride*
Th	*Trichoderma harzianum*
Sc	*Schizophyllum commune* Xylanase A
An	*Aspergillus niger*, var. *awamori*
At	*Aspergillus tubigensis*
Tr1	*Trichoderma reesei* XYN I
Ss	*Streptomyces* sp. 36a
S1B	*Streptomyces lividans* Xln B
S1C	*Streptomyces lividans* Xln C
Tf	*Thermomonospora fusca* TfxA
Bc	*Bacillus circulans*
Bs	*Bacillus subtilis*

Aligned amino acid sequences numbering from N-terminus

First section

Lowest Amino acid #		Highest amino acid #	
Ca	23	S AFNTQAAP	31
Cs	1	G	1

Second Section

Lowest amino acid #					Highest amino acid #	
Bp	1	RTITNNEMGN	HSGYDYELWK	DYGNT-SMTL	NNGGAFSAGW	N--NIGNA 45
Ca	32	KTITSNEIGV	NGGYDYELWK	DYGNT-SMTL	KNGGAFSCQW	S--NIGNA 76
Cs	2	RIIYDNETGT	HGGYDYELWK	DYGNT-IMEL	NDGGTFSCQW	S--NIGNA 46
Rf	1	SAADQQTRGN	VGGYDYEMWN	QNGQGQASMN	PGAGSFTCSW	S--NIENF 46
Tr2	1	QTIQPGTGY	NNGYFYSYWN	DGHGGVTYTN	GPGGQFSVNW	S--NSGNF 45
Tv	1	QTIQPGTGF	NNGYFYSYWN	DGHGGVTYTN	GPGGQFSVNW	S--NSGNF 45
Th	1	QTIGPGTGY	SNGYYYSYWN	DGHAGVTYTN	GGGGSFTVNW	S--NSGNF 45
Sc	1	SGTPSSTGT	DGGYYYSWWT	DGAGDATYQN	NGGGSYTLTW	SG-NNGNL 46
An	1	S	AGINYVQNYN	GNLGDFTY-D	ESAGTFSMYW	EDGVSSDF 37
AT	1	S	AGINYVQNYN	QNLGDFTY-D	ESAGTFSMYW	EDGVSSDF 37
Tr1	1		ASINYDQNYQ	TGG-QVSYS-	PSNTGFSVNW	N--TQDDF 34
Ss	1	ATTIT-NETGY	D-GMYYSFWT	DGGGSVSMTL	NGGGSYSTRW	T--NCGNF 45
S1B	1	DTVVTTNQEGT	NNGYYYSFWT	DSQGTVSMNM	GSGGQYSTSW	R--NTGNF 47
S1C	1	ATTITTNQTGT	D-GMYYSFWT	DGGGSVSMTL	NGGGSYSTQW	T--NCGNF 46
Tf	1	AVTSNETGY	HDGYFYSFWT	DAPGTVSMEL	GPGGNYSTSW	R--NTGNF 45
Bc	1		ASTDYWQNWT	DGGGIVNAVN	GSGGNYSVNW	S--NTGNF 36
Bs	1		ASTDYWQNWT	DGGGIVNAVN	GSGGNYSVNW	S--NTGNF 36

Figure 1 (continued)

Third section

	Lowest amino acid #				Highest amino acid #
Bp	46	LFRK-GKKFD ST-RTHHQLG	NISINYNASF	N-PSGNSYLC	VXGWTQSP 90
Ca	77	LFRK-GKKFN DT-QTYKQLG	NISVNYNCNY	Q-PYGNSYLC	VXGWTSSP 121
Cs	47	LFRK-GRKFN SD-KTYQELG	DIVVEYGCDY	N-PNGNSYLC	VXGWTRNF 91
Rf	47	LARM-GKNYD SQKKNYKAFG	NIVLTYDVEY	T-PRGNSYMC	VXGWTRNP 92
Tr2	46	VGGK-GWQPG TKNKV-----	---INFS-GS	YNPNGNSYLS	VXGWSRNP 83
Tv	46	VGGK-GWQPG TKNKV-----	---INFS-GS	YNPNGNSYLS	VXGWSRNP 83
Th	46	VGGK-GWQPG TKNKV-----	---INFS-GS	YNPNGNSYLS	IXGWSRNP 83
Sc	47	VGGK-GWNPG AASRS-----	---ISYS-GT	YQPNGNSYLS	VXGWTRSS 84
An	38	VVGL-GWTTG SSNA------	---ITYSAEY	SASGSSSYLA	VXGWVNYP 76
At	38	VVGLGGWTTG SSNA------	---ITYSAEY	SASGSASYLA	VXGWVNYP 76
Tr1	35	VVGV-GWTTG SSAP------	---INFGGSF	SVNSGTGLLS	VXGWSTNP 72
Ss	46	VAGK-GWANG GR-RT-----	---VRYT-GW	FNPSGNGYGC	LXGWTSNP 82
S1B	48	VAGK-GWANG GR-RT-----	---VQYS-GS	FNPSGNAYLA	LXGWTSNP 84
S1C	47	VAGK-GWSTG DGN-------	---VRYN-GY	FNPVGNGYGC	LXGWTSNP 82
Tf	46	VAGK-GWATG GR-RT-----	---VTYS-AS	FNPSGNAYLT	LXGWTRNP 82
Bc	37	VVGK-GWTTG SPFRT-----	---INYNAGV	WAPNGNGYLT	LXGWTRSP 75
Bs	37	VVGK-GWITG SPFRT-----	---INYNAGV	WAPNGNGYLT	LXGWTRSP 75

Fourth section

	Lowest amino acid #				Highest amino acid #
Bp	91	LAEXXIVDSW GTYR-PT--G	AYKGSFYADG	GTXDIYETTR	VNQPSIIG 135
Ca	122	LVEXXVIDSW GSWRPP--GG	TSKGTITVDG	GIXDIYETTR	INQPSIQG 167
Cs	92	LVEXXIVESW GSWRPP--GA	TPKGTITQWMAGTXEIYETTR		VNQPSIDG 138
Rf	93	LMEXXIVEGW GDWRPPGNDG	EVKGTVSANG	NTXDIRKTMR	YNQPSLDG 140
Tr2	84	LIEXXIVENF GTYN-PSTGA	TKLGEVTSDG	SVXDIYRTQR	VNQPSIIG 130
Tv	84	LIEXXIVENF GTYN-PSTGA	TKLGEVTSDG	SVXDIYRTQR	VNQPSIIG 130
Th	84	LIEXXIVENF GTYN-PSTGA	TKLGEVTSDG	SVXDIYRTQR	VNQPSIIG 130
Sc	85	LIEXXIVESY GSYD-PSSAA	SHKGSVTCNG	ATXDILSTWR	YNAPSIDG 131
An	77	GAEXXIVEDY GDYN-PCSSA	TSLGTVYSDG	STXQVCTDTR	INEPSITG 123
At	77	QAEXXIVEDY GDYN-PCSSA	TSLGTVYSDG	STXQVCTDTR	INEPSITG 123
Tr1	73	LVEXXIMEDN HNY--PAQ-G	TVKGTVTSDG	ATXTIWENTR	VNEPSIQG 117
Ss	83	LVEXXIVDNW GSYR-PT--G	ETRGTVHSDG	GTXDIYKTTR	YNAPSVEA 127
S1B	85	LVEXXIVDNW GTYR-PT--G	EYKGTVTSDG	GTXDIYKTTR	VNKPSVEG 129
S1C	83	LVEXXIVDNW GSYR-PT--G	TYKGTVSSDG	GTXDIYQTTR	YNAPSVEG 127
Tf	83	LVEXXIVESW GTYR-PT--G	TYMGTVTTDG	GTXDIYKTTR	YNAPSIEG 127
Bc	76	LIEXXVVDSW GTYR-PT--G	TYKGTVKSDG	GTXDIYTTTR	YNAPSIDG 120
Bs	76	LIEXXVVDSW GTYR-PT--G	TYKGTVKSDG	GTXDIYTTTR	YNAPSIDG 120

Figure 1 (continued)

Fifth section

	Lowest amino acid #				Highest amino acid #
Bp	136	-IATFKQYWE	VRQTKRTS--	------GTVS	VSAHFRKWES LGMPM-GK 173
Ca	168	-NTTFKQYWE	VRRTKRTS--	------GTIS	VSKHFAAWES KGMPL-GK 206
Cs	139	-TATFQQYWE	VRTSKRTS--	------GTIS	VTEHFKQWER MGMRM-GK 177
Rf	141	-TATFPQYWE	VRQTSGSANN	QTNYMKGTID	VSKHFDAWSA AGLDMSGT 187
Tr2	131	-TATFYQYWE	VRRNHR-S-S	------GSVN	TANHFNAWAQ QGLTL-GT 168
Tv	131	-TATFYQYWE	VRRTHR-S-S	------GSVN	TANHFNAWAQ QGLTL-GT 168
Th	131	-TATFYQYWE	VRRNHR-S-S	------GSVN	TANHFNAWAS HGLTL-GT 168
Sc	132	-TQTFEQFWE	VRNPKKAPGG	SIS---GTVD	VQCHFDAWKG LGMNLGSE 175
An	124	-TSTFTQYFE	VRESTRTS--	------GTVT	VANHFNFWAQ HGFGN-SD 162
At	124	-TSTFTQYFE	VRESTRTS--	------GTVT	VANHFNFWAH HGFHN-SD 162
Tr1	118	-TATFNQYIE	VRNSPR-T-S	------GTVT	VQNHFN-WAS LGLHLGQM 155
Ss	128	-PAAFDQYWE	VRQSKVT--S	------GTIT	TGNHFDAWAR AGMNMGNF 166
SlB	130	TR-TFDQYWE	VRQSKR-TG-	------GTIT	TGNHFDAWAR AGMPLGNF 168
SlC	128	TK-TFQQYWE	VRQSKVTSGS	------GTIT	TGNHFDAWAR AGMNMGQF 168
Tf	128	TR-TFDQYWE	VRQSKRTS--	------GTIT	AGNHFDAWAR HGMHLGTH 166
Bc	121	DRTTFTQYWE	VRQSKRPTGS	N------ATIT	FTNHVNAWKS HGMNLGSN 163
Bs	121	DRTTFTQYWE	VRQSKRPTGS	N------ATIT	FSNHVNAWKS HGMNLGSN 163

Sixth section

	Lowest amino acid #			Highest amino acid #
Bp	174	MYETAFTVEG	YQSSGSANVM	TNQLFIGN 201
Ca	207	MHETAFNIEG	YQSSGKADVN	SMSINIGK 233
Cs	178	MYEVALTVEG	YQSSGYANVY	KNEIRIGANP....
Rf	188	LYEVSLNIEG	YRSNGSANVK	SVSV 211
Tr2	169	MDYQIVAVEG	YFSSGSASI-	TVS 190
Tv	169	MDYQIVAVEG	YFSSGSASI-	TVS 190
Th	169	MDYQIVAVEG	YFSSGSASI-	TVS 190
Sc	176	HNYQIVATEG	YQSSGTATI-	TVT 197
An	163	FNYQVMAVEA	WSGAGSASV-	TISS 184
At	163	FNYQVVAVEA	WSGAGSAAV-	TISS 184
Tr1	156	MNYQVVAVEG	WGGSGSASQ-	SVSN 178
Ss	167	RYYMINATEG	YQSSGSSTI-	TVSG 189
SlB	169	SYYMINATEG	YQSSGTSSI-	NVGG.....
SlC	169	RYYMINATEG	YQSSGSSNI-	TVSG 191
Tf	167	D-YMIMATEG	YQSSGSSNVT	LGTS.....
Bc	164	WAYQVMATEG	YQSSGSSNV-	TVW 185
Bs	164	WAYQVMATEG	YQSSGSSNV-	TVW 185

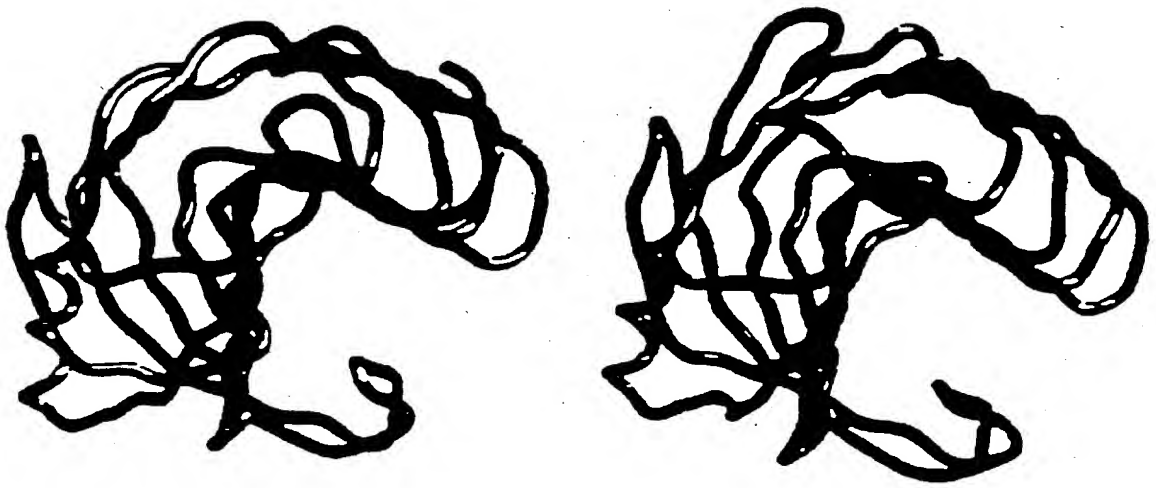

Figure 2

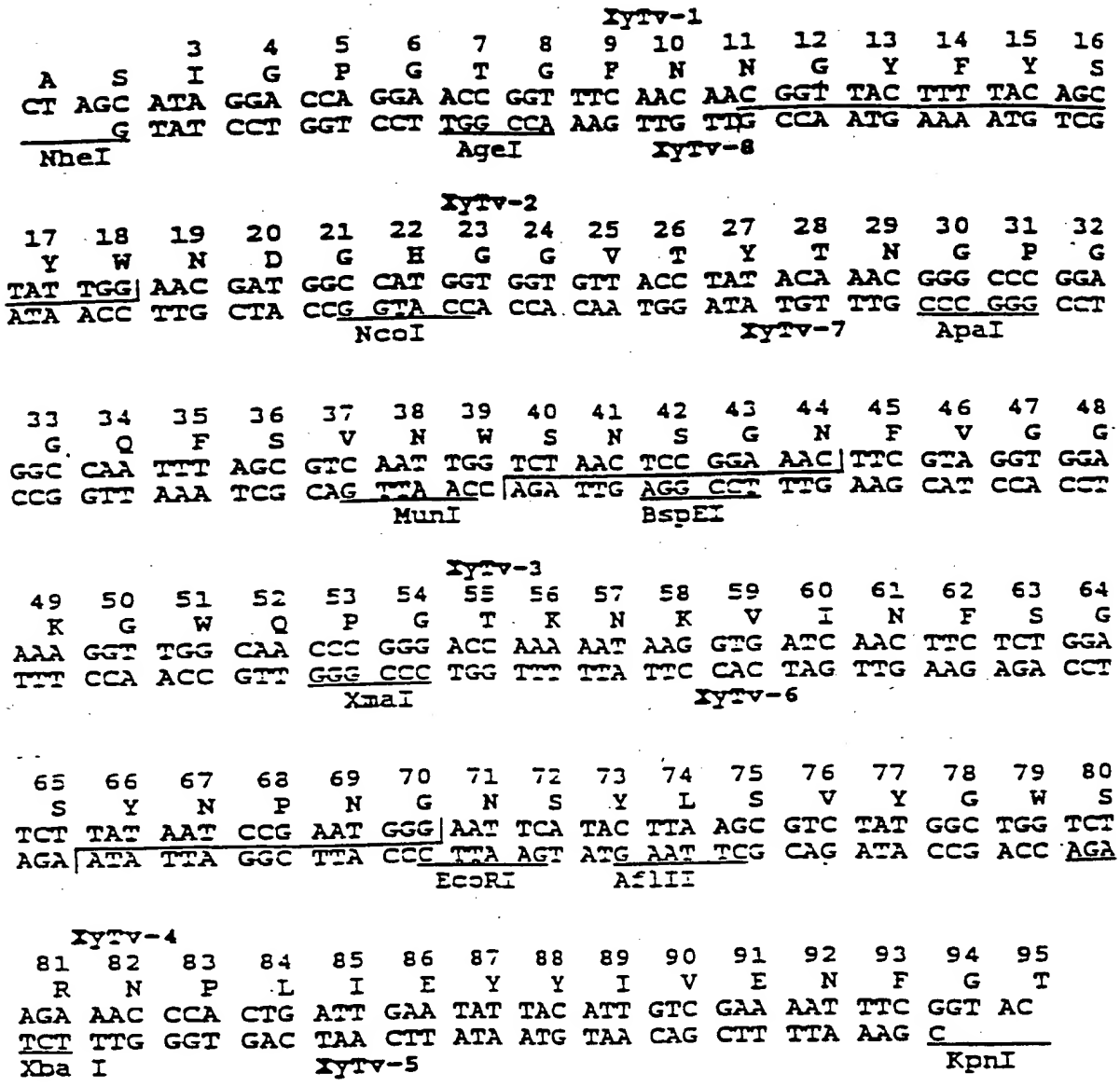

FIGURE 3

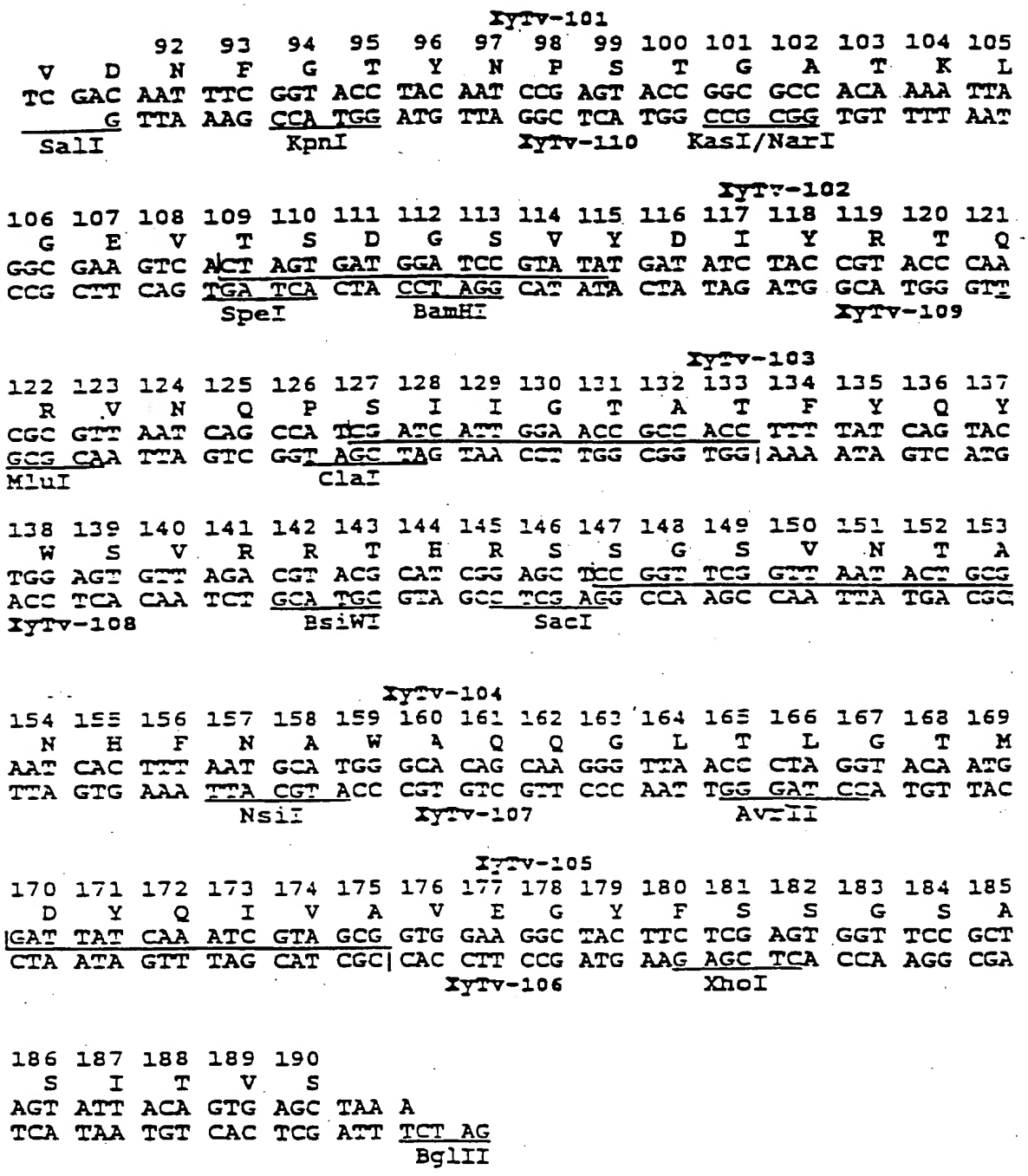

FIGURE 3 CONT'D

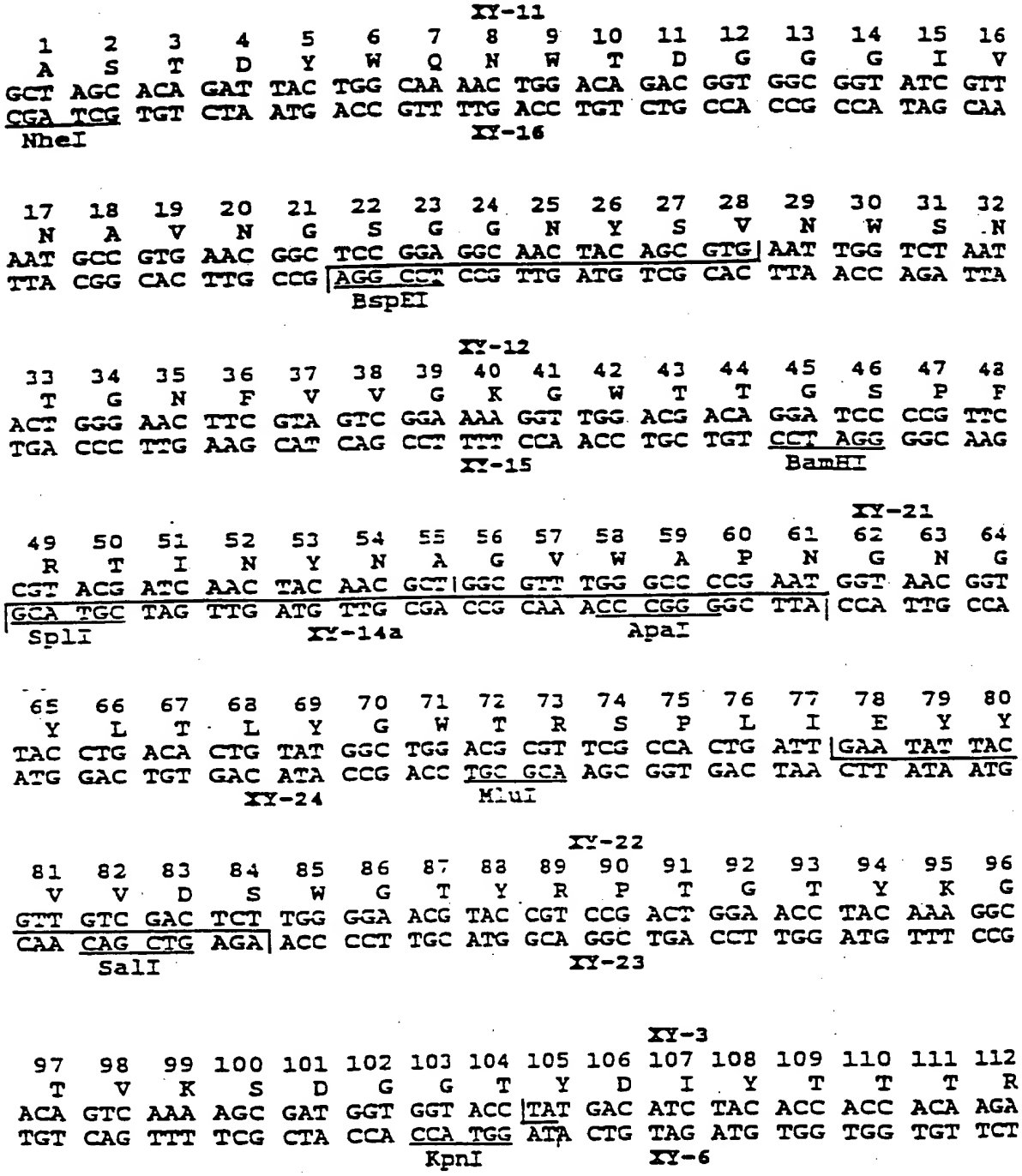

FIGURE 4

```
113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128
  Y   N   A   P   S   I   D   G   D   R   T   T   F   T   Q   Y
TAC AAC GCA CCT TCC ATC GAT GGC GAT CGG ACC ACC TTT ACT CAG TAT
ATG TTG CGT GGA AGG TAG CTA CCG CTA GCC TGG TGG AAA TGA GTC ATA
                    ClaI

129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144
  W   S   V   R   Q   S   K   R   P   T   G   S   N   A   T   I
TGG AGT GTT AGA CAA TCT AAG CGG CCG ACT GGT TCG AAC GCC ACC ATT
ACC TCA CAA TCT GTT AGA TTC GCC GGC TGA CCA AGC TTG CGG TGG TAA
                        EagI        BstBI

     XY-2
145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160
  T   F   T   N   H   V   N   A   W   K   S   H   G   M   N   L
ACG TTC ACC AAT CAC GTG AAT GCA TGG AAA TCC CAC GGT ATG AAC CTA
TGC AAG TGG TTA GTG CAC TTA CGT ACC TTT AGG GTG CCA TAC TTG GAT
    XY-5                    NsiI                        AvrII

161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176
  G   S   N   W   A   Y   Q   V   M   A   T   E   G   Y   Q   S
GGT TCT AAT TGG GCT TAT CAA GTA ATG GCG ACC GAA GGC TAC CAG AGC
CCA AGA TTA ACC CGA ATA GTT CAT TAC CGC TGG CTT CCG ATG GTC TCG
                XY-4b                                   SacI

                XY-1
177 178 179 180 181 182 183 184 185
  S   G   S   S   N   V   T   V   W
TCT GGT TCT TCC AAC GTT ACA GTG TGG TAA AGA TCT TGA AGC TTGGGACGT
AGA CCA AGA AGG TTG CAA TGT CAC ACC ATT TCT AGA ACT TCG AACCC
                XY-4a               BglII   HindIII   AatII
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FIGURE 4 CONT'D

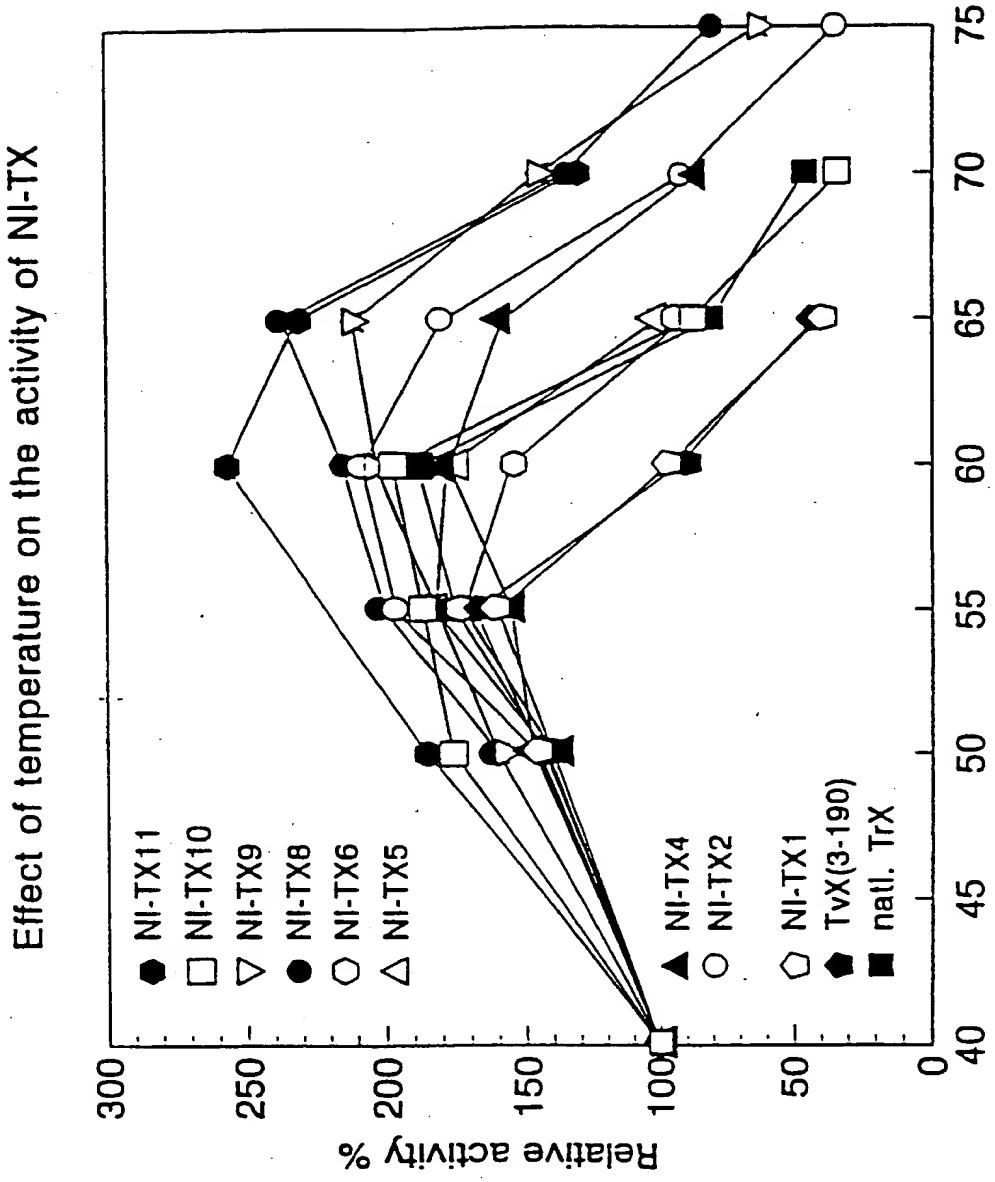

FIGURE 5

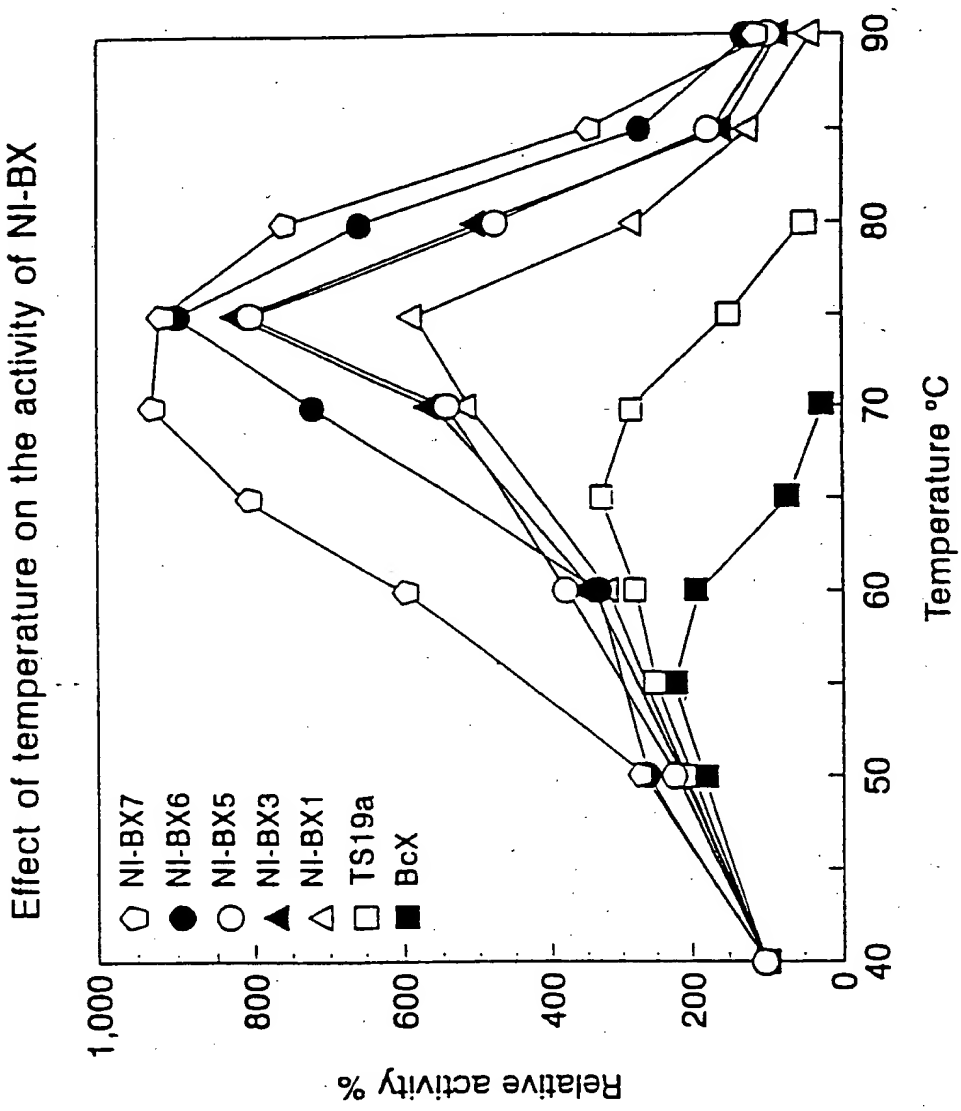
Effect of temperature on the activity of NI-BX
NI-BX7
NI-BX6
NI-BX5
NI-BX3
NI-BX1
TS19a
BcX
Relative activity %
1,000
800
600
400
200
0
40
50
60
70
80
90
Temperature °C

FIGURE 6

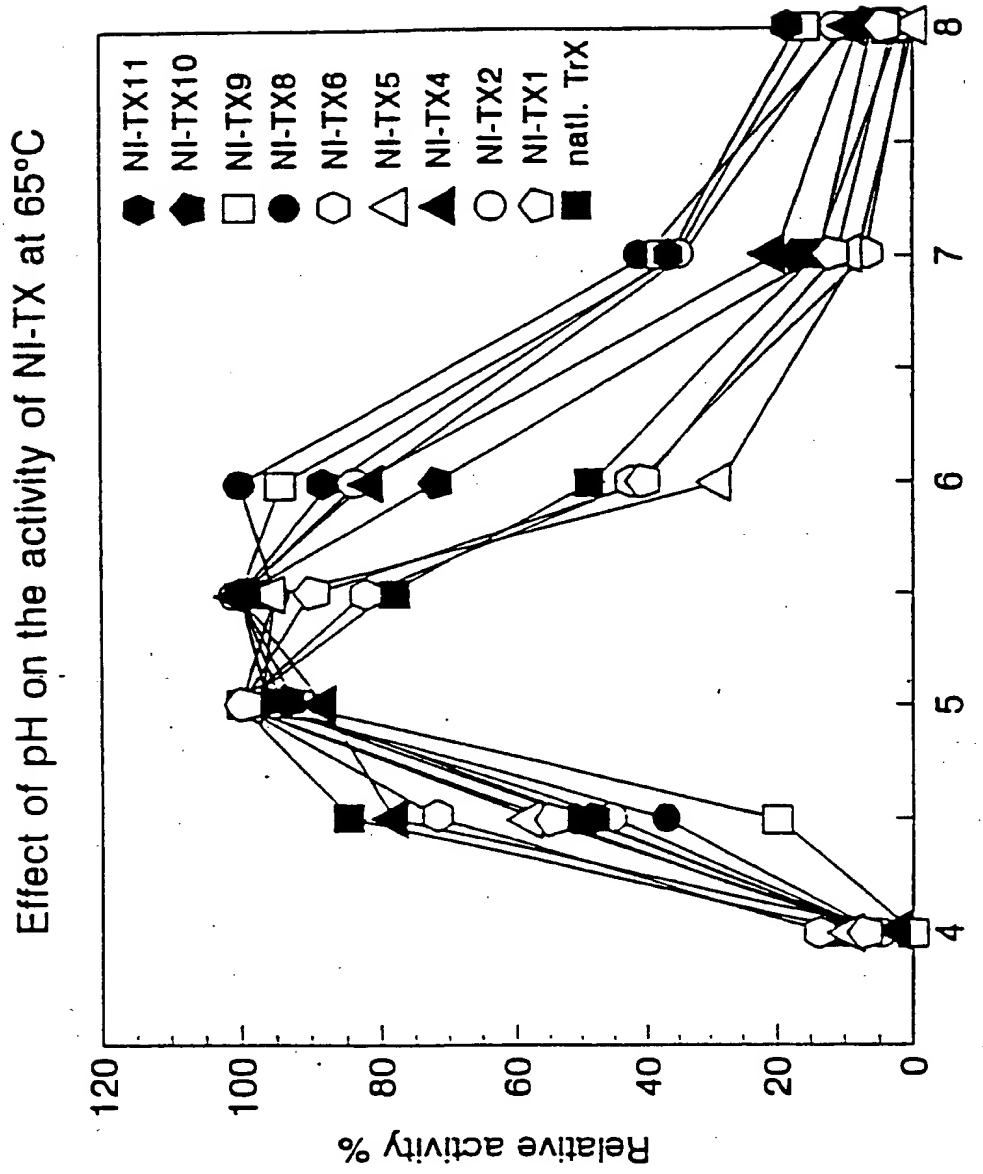

FIGURE 7

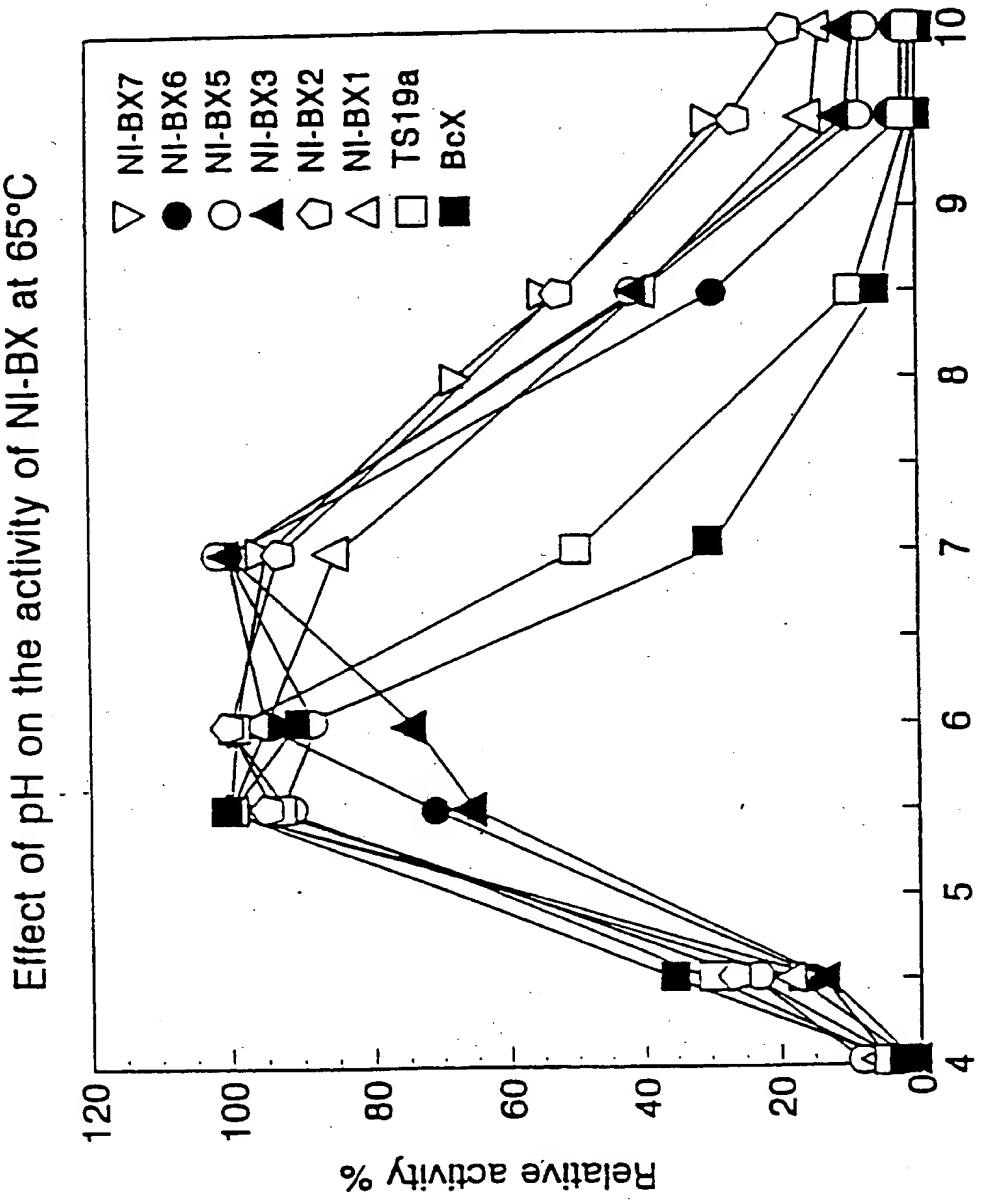
Effect of pH on the activity of NI-BX at 65°C
NI-BX7
NI-BX6
NI-BX5
NI-BX3
NI-BX2
NI-BX1
TS19a
BcX
Relative activity %
120
100
80
60
40
20
0
4
5
6
7
8
9
10
pH

FIGURE 8

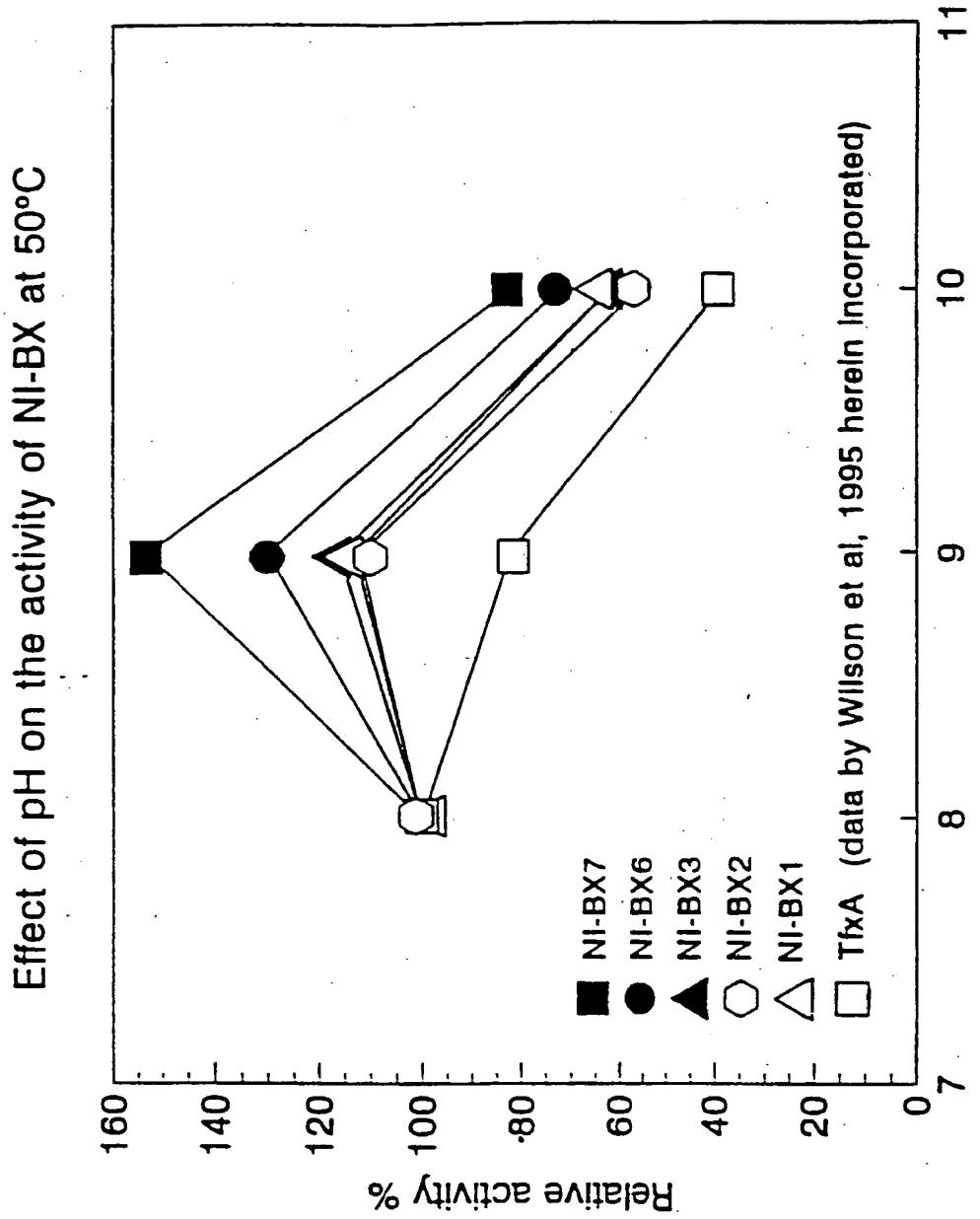

FIGURE 9

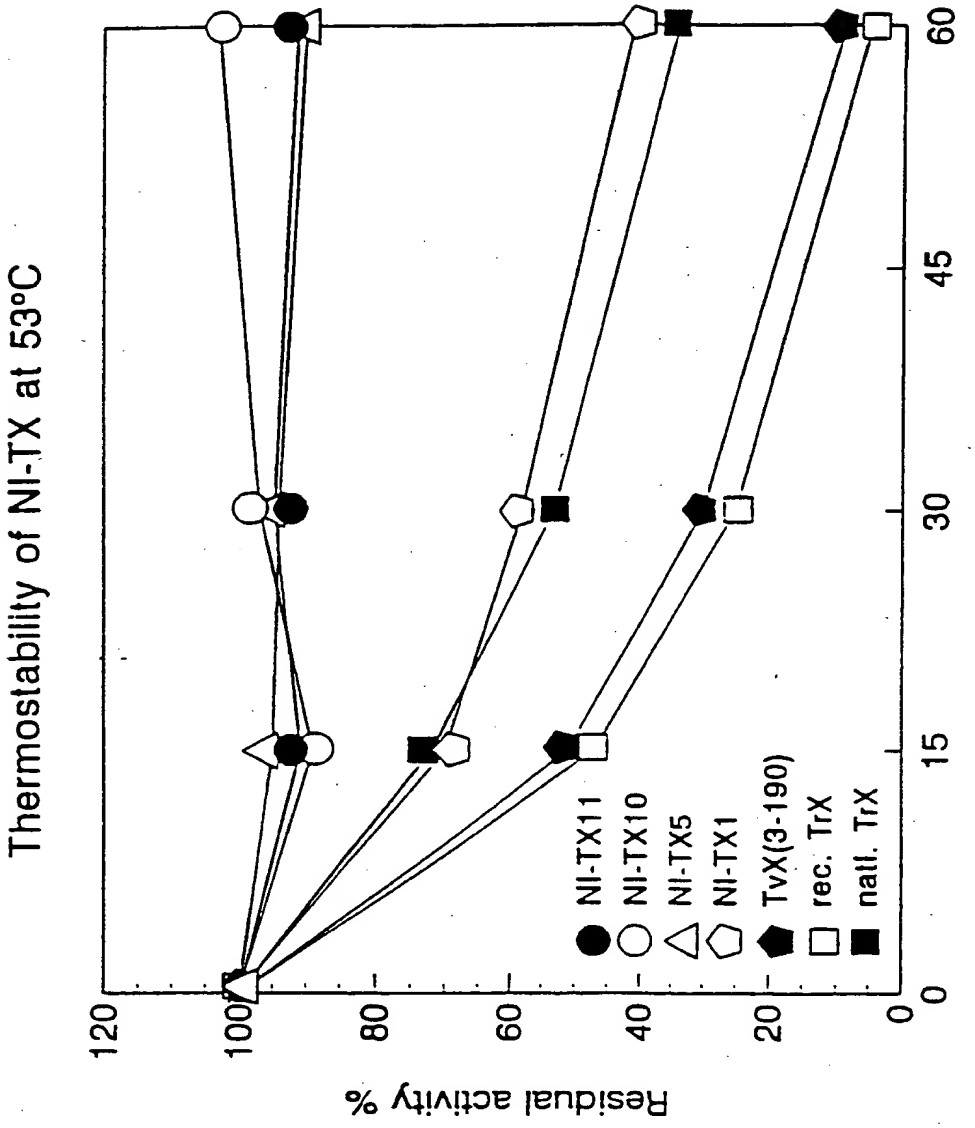
Thermostability of NI-TX at 53°C
Residual activity %
120
100
80
60
40
20
0
0
15
30
45
60
Incubation time (min)
NI-TX11
NI-TX10
NI-TX5
NI-TX1
TvX(3-190)
rec. TrX
natl. TrX

FIGURE 10

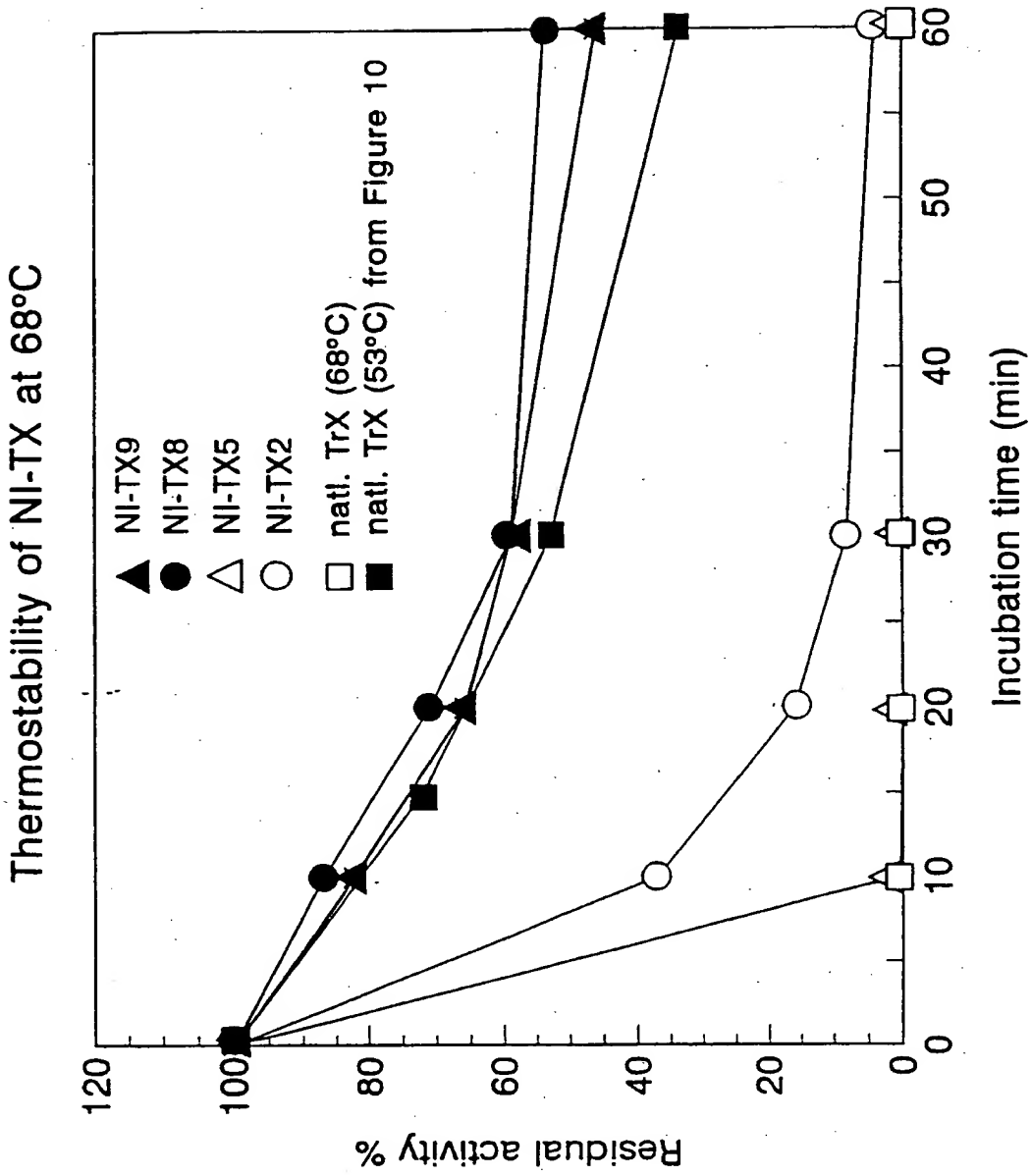

FIGURE 11

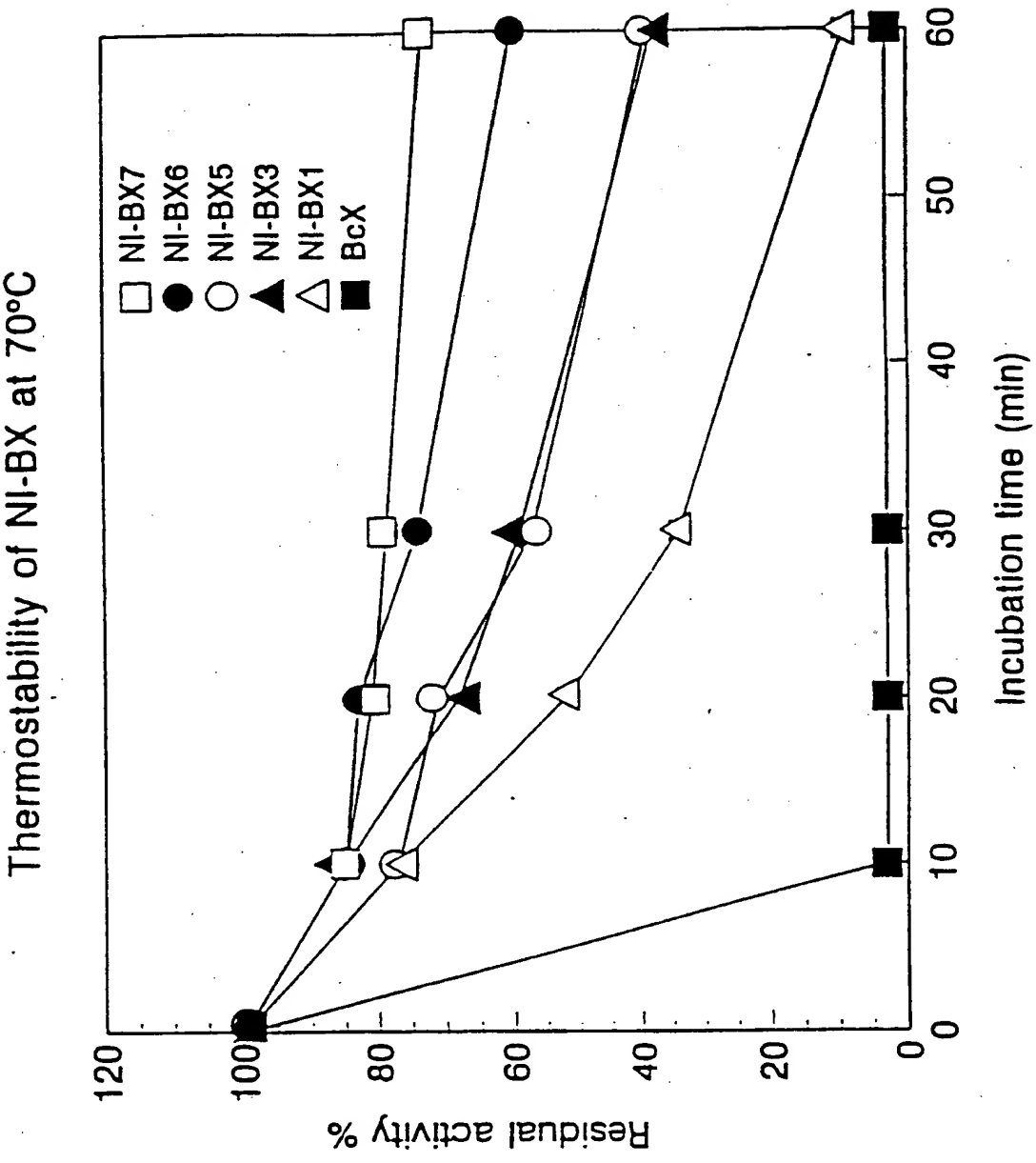
Thermostability of NI-BX at 70°C
NI-BX7
NI-BX6
NI-BX5
NI-BX3
NI-BX1
BcX
Residual activity %
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Incubation time (min)

FIGURE 12

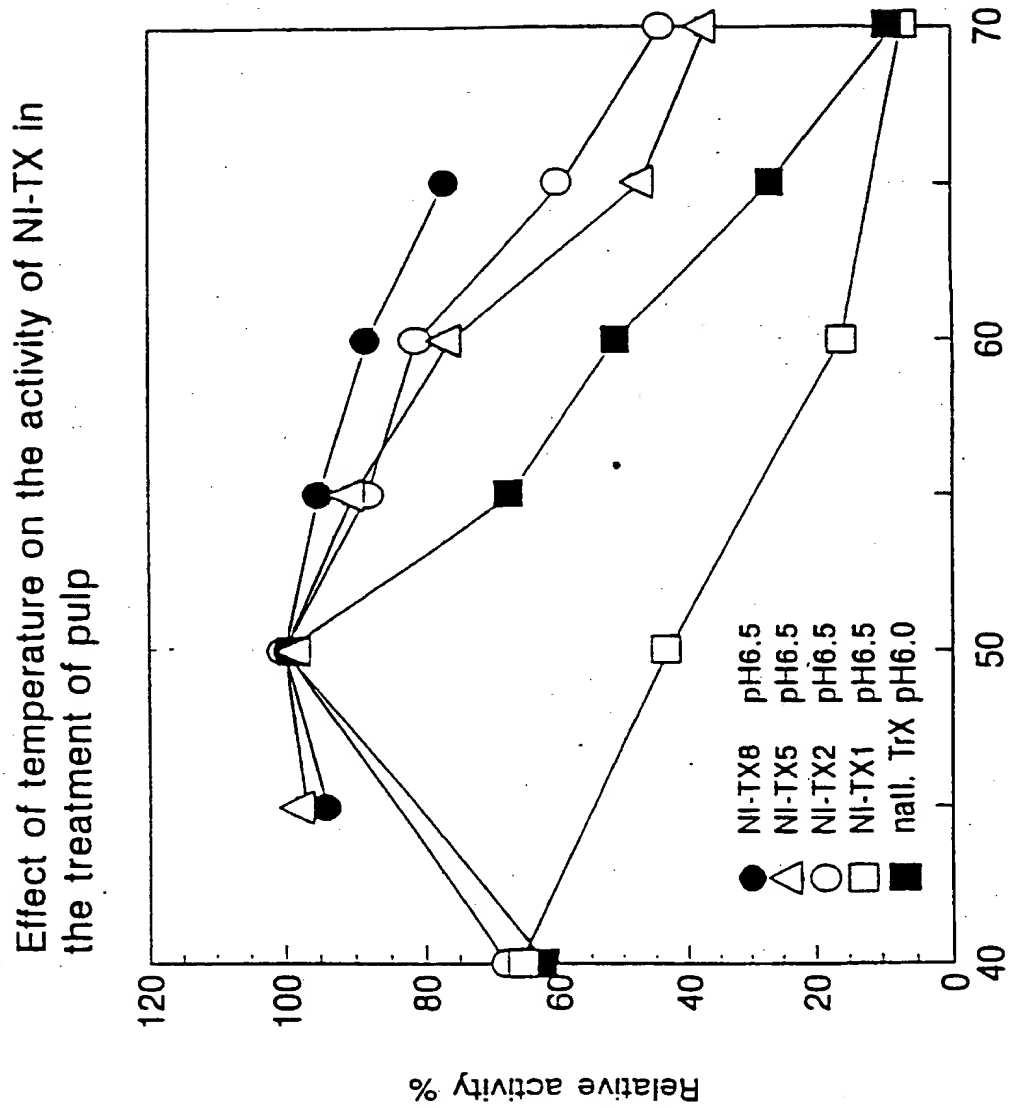

FIGURE 13

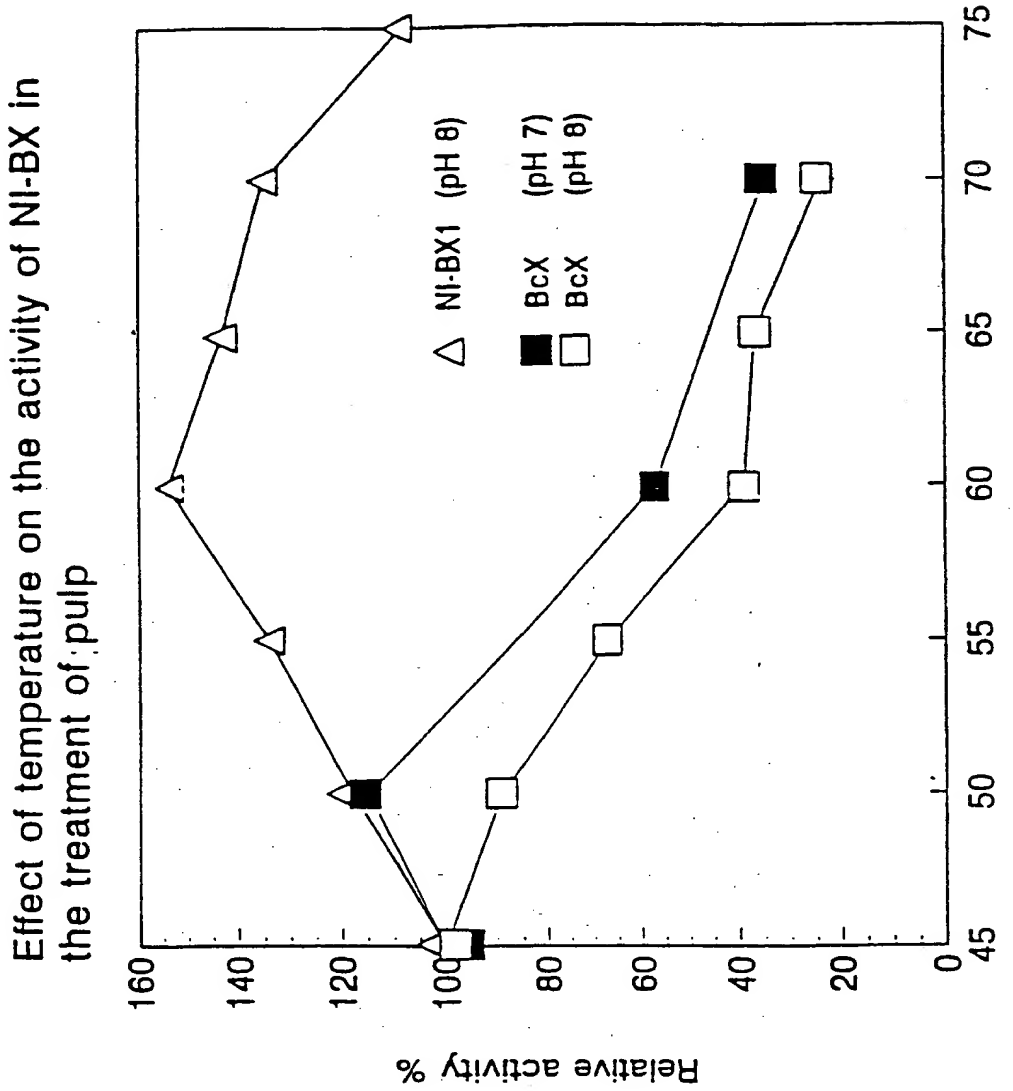
Effect of temperature on the activity of NI-BX in the treatment of pulp
Relative activity %
160
140
120
100
80
60
40
20
0
45
50
55
60
65
70
75
Temperature (°C)
NI-BX1 (pH 8)
BcX (pH 7)
BcX (pH 8)

FIGURE 14

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(71) Applicant:
NATIONAL RESEARCH COUNCIL OF CANADA
Ottawa Ontario K1A OR6 (CA)

(72) Inventors:
- **Sung, Wing L.**
  Gloucester, Ontario K1J 6A4 (CA)
- **Yaguchi, Makoto**
  Ottawa, Ontario, K2C 3N5 (CA)
- **Ishikawa, Kazuhiko**
  Tsukuba City, Ibaragi-Ken, 305 (JP)

(74) Representative:
von Kreisler, Alek, Dipl.-Chem. et al
Patentanwälte,
von Kreisler-Selting-Werner,
Bahnhofsvorplatz 1 (Deichmannhaus)
50667 Köln (DE)

(54) Modification of xylanase to improve thermophilicity, alkophilicity and thermostability

(57) Producing a xylanase enzyme of superior performance in the bleaching of pulp. More specifically, a modified xylanase of Family 11 that shows improved thermophilicity, alkalophilicity, and thermostability as compared to the natural xylanase. The modified xylanases contain any of three types of modifications: (1) changing amino acids 10, 27, and 29 of *Trichoderma reesei* xylanase II or the corresponding amino acids of another Family 11 xylanase, where these amino acids are changed to histidine, methionine, and leucine, respectively; (2) substitution of amino acids in the N-terminal region with amino acids from another xylanase enzyme. In a preferred embodiment, substitution of the natural *Bacillus circulans* or *Trichoderma reesei* xylanase with a short sequence of amino acids from *Thermomonospora fusca* xylanase yielded chimeric xylanases with higher thermophilicity and alkalophilicity; (3) an extension upstream of the N-terminus of up to 10 amino acids. In a preferred embodiment, extension of the N-terminus of the xylanase with the tripeptide glycine-arginine-arginine improved its performance.

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Application Number
EP 97 11 5412

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	MOREAU A ET AL: "Increase in catalytic activity and thermostability of the xylanase A of Streptomyces lividans 1326 by site-specific mutagenesis." ENZYME AND MICROBIAL TECHNOLOGY 16 (5). 420-424. ISSN: 0141-0229, May 1994, XP002072550 * the whole document * ---		C12N15/56 C12N9/24 D21C9/10 C12N15/62
A	SUNG W L ET AL: "Expression of Trichoderma reesei and Trichoderma viride xylanases in Escherichia coli." BIOCHEMISTRY AND CELL BIOLOGY ISSN: 0829-8211, vol. 73, no. 5 & 6, May 1995 - June 1995, pages 253-259, XP002072551 * the whole document * ---		
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	24 July 1998	Hix, R

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The present search report has been drawn up for all claims

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P,Y	TORRONEN A ET AL: "Structural and functional properties of low molecular weight endo-1,4-beta- xylanases." JOURNAL OF BIOTECHNOLOGY 57 (1-3). 1997. 137-149. ISSN: 0168-1656, XP004097716 * the whole document * ---	1-25	
P,Y	SAMAIN E ET AL: "High level production of a cellulase-free xylanase in glucose-limited fed batch cultures of a thermophilic Bacillus strain." JOURNAL OF BIOTECHNOLOGY 58 (2). 1997. 71-78. ISSN: 0168-1656, XP002072557 * the whole document * -----	1-25	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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